SHOUT
SHARE
IT

# THE

# BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

VOLUME 104
FEBRUARY TO JUNE, 1953

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

---

LANCASTER PRESS, INC., LANCASTER, PA.

# CONTENTS

No. 1. February, 1953

PAGE

Bodine, Joseph Hall, and William Lionel West
Carbohydrate metabolism of the developing egg and embryo .......... 1
Child, C. M.
Indicator gradient patterns in oöcytes and early developmental stages of echinoderms: A reexamination .................................. 12
Hiatt, Robert W., John J. Naughton and Donald C. Matthews
Effects of chemicals on a schooling fish, Kuhlia sandvicensis ........... 28
Kempton, Rudolf T.
Studies on the elasmobranch kidney. II. Reabsorption of urea by the smooth dogfish, Mustelus canis .................................. 45
Mazia, Daniel, Philip A. Brewer and Max Alfert
The cytochemical staining and measurement of protein with mercuric bromphenol blue .................................. 57
Moore, John A., and Betty C. Moore
Studies in the development of frog hybrids. IV. Competence of gastrula ectoderm in androgenetic hybrids .................................. 68
Scholander, P. F., and L. Van Dam
Composition of the swimbladder gas in deep sea fishes ................ 75
Sealander, John A., Jr.
Temperatures of white-footed mice in relation to environmental temperature and heat and cold stress .................................. 87
Stadler, David R.
Chemotropism in Rhizopus nigricans. II. The action of plant juices .... 100
Sze, L. C.
Respiratory gradients in Tubularia .................................. 109

No. 2. April, 1953

Guyselman, J. Bruce
An analysis of the molting process in the fiddler crab, Uca pugilator .... 115
Kelly, Sally
Respiration and iodine uptake in Ascophyllum ...................... 138
Loosanoff, Victor L.
Reproductive cycle in Cyprina islandica .......................... 146
Maynard, Donald M., Jr.
Activity in a crustacean ganglion. I. Cardio-inhibition and acceleration in Panulirus argus .................................. 156

67870

PAGE

RAO, K. PAMPAPATHI
Rate of water propulsion in Mytilus californianus as a function of latitude 171

RATTENBURY, JOAN C.
Reproduction in Phoronopsis viridis. The annual cycle in the gonads, maturation and fertilization of the ovum ........ 182

RUGH, ROBERTS
The x-irradiation of marine gametes. A study of the effects of x-irradiation at different levels on the germ cells of the clam, Spisula (formerly Mactra) ........ 197

SUGIYAMA, MASAO
Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. I. Response to sodium choleinate and wasp venom 210

SUGIYAMA, MASAO
Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. II. The propagating or non-propagating nature of the cortical changes induced by various reagents ........ 216

TYLER, ALBERT
Prolongation of life-span of sea urchin spermatozoa, and improvement of the fertilization-reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron) 224

VALLOWE, HENRY H.
Some physiological aspects of reproduction in Xiphophorus maculatus ... 240

WHEELER, AMBROSE J.
Temporal variations in histological appearance of thyroid and pituitary of salamanders treated with thyroid inhibitors ........ 250

No. 3. JUNE, 1953

BARNETT, ROBERT C.
Cell division inhibition of Arbacia and Chaetopterus eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds ........ 263

BLISS, DOROTHY E.
Endocrine control of metabolism in the land crab, Gecarcinus lateralis (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs ........ 275

BONNER, JOHN TYLER, AND EVELYN BARBARA FRASCELLA
Variations in cell size during the development of the slime mold, Dictyostelium discoideum ........ 297

VON BRAND, THEODOR, AND BENJAMIN MEHLMAN
Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some fresh water snails ........ 301

CARLSON, J. GORDON, NYRA G. HARRINGTON AND MARY ESTHER GAULDEN
Mitotic effects of prolonged irradiation with low-intensity gamma rays on the Chortophaga neuroblast ........ 313

CHADWICK, L. E., J. B. LOVELL AND V. E. EGNER
The effect of various suspension media on the activity of cholinesterase from flies ........ 323

PAGE
DAVIS, HARRY C.
On food and feeding of larvae of the American oyster, C. virginica ...... 334
EICHEL, HERBERT J., AND JAY S. ROTH
The effect of x-irradiation on nuclease activity and respiration of Tetrahymena geleii W. ........ 351
FRAENKEL, G.
Studies on the distribution of vitamin $B_T$ (carnitine) ........ 359
GRÉGOIRE, CH.
Blood coagulation in arthropods. III. Reactions of insect hemolymph to coagulation inhibitors of vertebrate blood ........ 372
HIRATA, ARTHUR A.
Studies on shell formation. II. A mantle-shell preparation for in vitro studies ........ 394
JODREY, LOUISE H.
Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$ ........ 398
METZ, CHARLES B., DOROTHY R. PITELKA AND JANE A. WESTFALL
The fibrillar systems of ciliates as revealed by the electron microscope. I. Paramecium ........ 408
PERRY, ALBERT S., ARNOLD M. MATTSON AND ANNETTE J. BUCKNER
The mechanism of synergistic action of DMC with DDT against resistant house flies ........ 426
SOTAVALTA, OLAVI
Recordings of high wing-stroke and thoracic vibration frequency in some midges ........ 439
VERNBERG, F. JOHN, AND I. E. GRAY
A comparative study of the respiratory metabolism of excised brain tissue of marine teleosts ........ 445

Vol. 104, No. 1 February, 1953

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

# CARBOHYDRATE METABOLISM OF THE DEVELOPING EGG AND EMBRYO[1]

JOSEPH HALL BODINE AND WILLIAM LIONEL WEST

*Zoology Laboratory, State University of Iowa, Iowa City, Iowa*

Studies on the respiration of intact eggs and isolated embryos of the grasshopper, *Melanoplus differentialis,* reveal that the functional intensity of the enzymes concerned with aerobic metabolism is altered considerably during different stages of embryonic development (Bodine and Boell, 1934; Bodine and Boell, 1936; Boell, 1935; Bodine and Lu, 1950a, 1950b; Bodine, Lu and West, 1952). The question of the nature and relative amounts of various metabolites used during embryonic development has attracted the attention of many investigators using a diversity of biological materials (Spratt, 1952). Needham (1942) has summarized large amounts of data and introduced a concept of a definite sequence in which metabolites are used during the course of embryonic development. It is also generally accepted that the terrestrial cleidoic egg consumes large amounts of fat and a much smaller amount of protein and carbohydrate during development. Results previously recorded in this laboratory seem to support these above views (Boell, 1935; Hill, 1945). Much of the evidence pertaining to metabolism during embryonic development has been obtained from data on the intact egg and little from studies of the intact embryo freed of yolk.

The importance of carbohydrates as energy sources in biological systems is well known. The present investigation is the first of a series planned to determine the importance of carbohydrate in the mechanism of the gross chemical transformations from yolk to embryo in the developing egg of the grasshopper.

## METHODS

Embryos of the grasshopper, *Melanoplus differentialis,* were obtained, yolk free, by previously described methods of Bodine and Boell (1934, 1936).

Fractionation of the embryos (Bodine and Lu, 1950a, 1951) was carried out either in Ringer solution buffered with 1/15 *M* phosphates (pH 6.8) or in 0.25 *M* sucrose containing 0.0035 *M* magnesium and calcium chlorides. No quantitative differences in results were observed between the two media and the latter was the one of choice since the morphological integrity of the intracellular particles seemed better preserved in it. The calcium ion in the sucrose medium was necessary to prevent clumping of nuclei and destruction of the nuclear membranes. The magnesium ion was found essential for maximum reactions of homogenates.

---

[1] Aided by a grant from the National Institutes of Health. Acknowledgment is gratefully made to Etta Andrews for technical assistance.

Respiration experiments were carried out using standard Warburg manometers. Seven experimental manometers contained 2,2,4-trimethyl pentane colored with Sudan IV as a manometer fluid and seven similar ones contained Brodie's solution. Both sets of manometers were carefully checked against each other. The sensitivity of the manometers was greatly increased by the use of the 2,2,4-trimethyl pentane as manometer fluid.

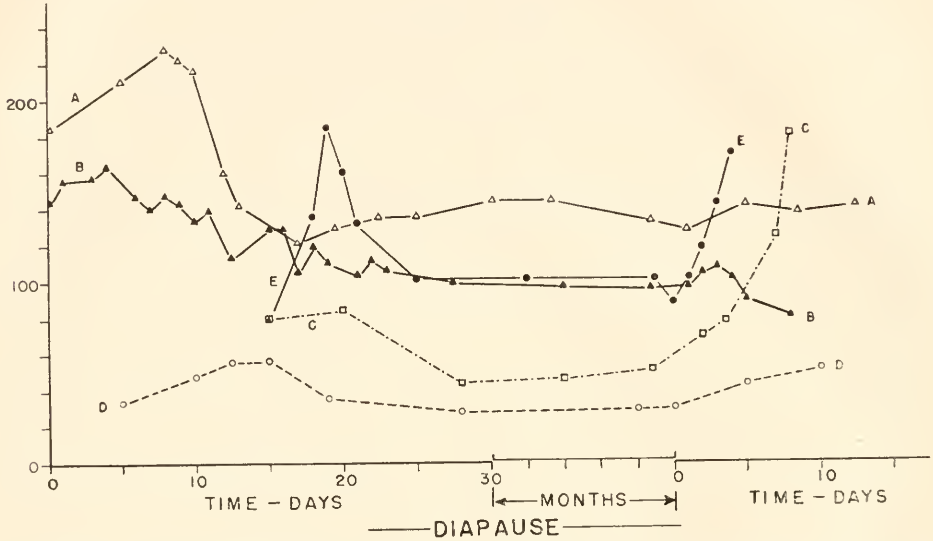


FIGURE 1. Shows total amount of carbohydrate per intact whole egg (A), and per yolk of whole egg (B); hexose content of embryo (C), and yolk (D), throughout course of embryonic development. Curve (E) shows endogenous $O_2$ uptake of embryo homogenates, taking that for the diapause embryo homogenate as 100%. Abscissa, time in days and months; ordinate micrograms per egg or embryo for carbohydrates, for $O_2$ uptake, percentage. Values for hexoses of embryo are increased by 1/10 over actual values found. Each point represents averages from many experiments.

Commercial glucose, glucose-1-phosphate (dipotassium salt), fructose-6-phosphate (dibarium salt converted to the sodium or potassium salt) and fructose 1,6-diphosphate (dibarium salt converted to sodium salt) obtained from the Swartz laboratories, were employed as substrates. The substrates (0.5 cc.) were tipped from the side arms of the manometer flasks at the end of a 40-minute control period. The final concentration of the substrates per 1.5 ml. (total volume of fluid in flask) was approximately 40 moles $\cong 0.026\ M$ for glucose, while fructose 1,6-diphosphate contained the same carbohydrate content per cc. as did glucose-1-phosphate.

Carbohydrate determinations were made with the commercial product "Microne" obtained from the National Biochemical Laboratory. "Microne" is chemically anthrone and was described by Dreywood (1946) as a specific test for carbohydrate. The maximum absorption of the green color developed using a glucose test solution and 0.2% "Microne" in 95% sulfuric acid was found to be 625 mμ which is similar to that reported by Viles and Silverman (1949). "Microne" is used as a microcolorimetric method for determination of sugars, starches and celluloses. This reagent is of interest since equivalent amounts of glucose, glycogen and fructose

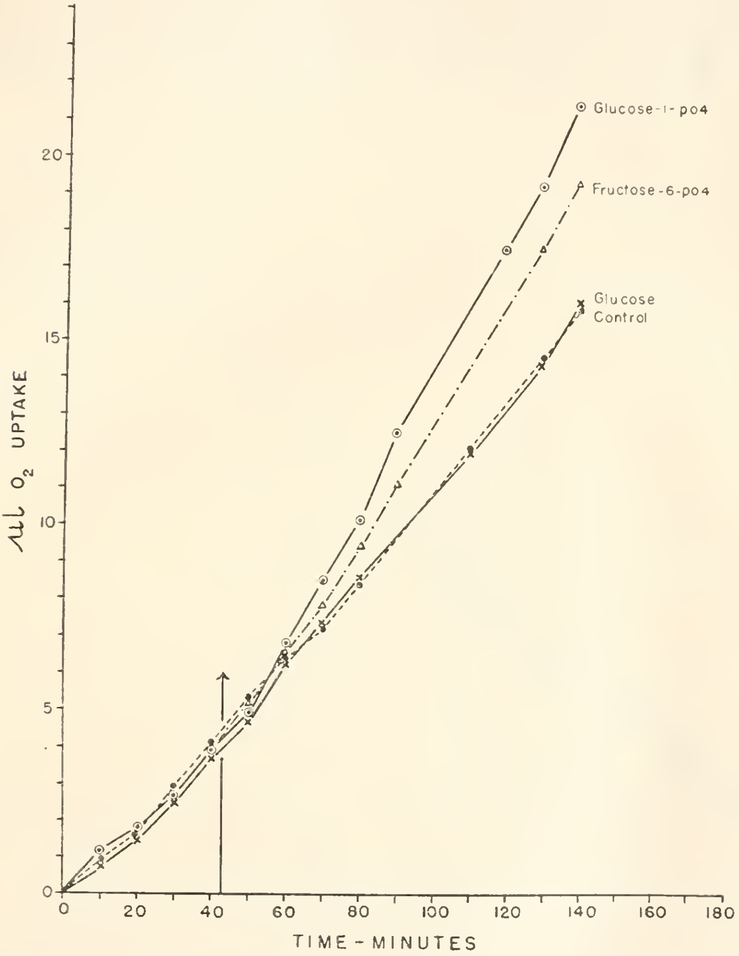


FIGURE 2. Shows endogenous $O_2$ uptake of diapause embryo suspended in different substrates. Arrow indicates addition of substrate. Abscissa, time in minutes; ordinate, micro liters of $O_2$ per egg. Graphs represent typical experiment.

give approximately the same amount (intensity) of color with the reagent (Morse, 1947; Morris, 1948).

To 3 cc. of an experimental solution, 6 cc. of "Microne" were added from a burette that permitted rapid flow of the viscous acid solution. The components of the tube were mixed rapidly and placed in a water bath (25° C. ± 0.2) 30 minutes for color development. At the end of this period, measurements were made immediately using the Coleman Model 1U spectrophotometer at 625 m$\mu$. A series of standards was run with each group of experimental solutions.

Analyses were made on each of the following: (a) An acid hydrolysis of the whole egg which is recorded as the total carbohydrate (Fig. 1, curve A). These data are strikingly similar to those found by Hill (1945). (b) A saline-soluble fraction which is recorded as the carbohydrate content of the yolk (Fig. 1, curve B). This latter fraction was obtained by suspending the contents of the egg in a concentration of 1 egg/2 cc., in 0.9% saline. The suspension was then centrifuged for one minute at low speed to remove embryo, shell and extra-embryonic membranes, after which an aliquot was taken for analysis. (c) A trichloracetic acid (TCA)-soluble fraction which is recorded separately as the hexose content of the embryo and the yolk (Fig. 1, curves C and D, respectively). The yolk hexose was obtained by breaking the eggs in 10% TCA and then removing the intact embryos, precipitated protein and shells by centrifugation (5000 G). Lipids were removed from centrifuged samples by means of suction pipettes. An aliquot of the supernatant was diluted and used for analysis and designated as yolk hexose. The embryo hexose was determined by homogenization of saline-washed embryos in 10% TCA. Centrifugation (5000 G) was employed to remove the precipitated protein and

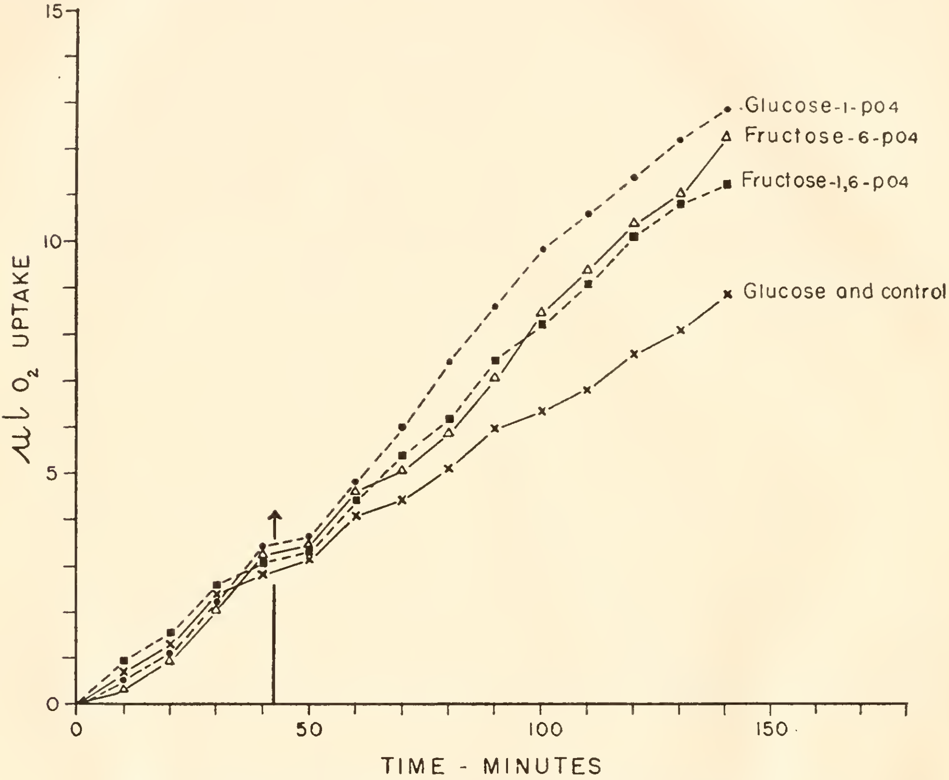


FIGURE 3. Same as Figure 2 except for homogenate of diapause embryo.

suction pipettes to remove the lipid layer. An aliquot was then diluted and used for analysis.

## RESULTS

The total carbohydrate content of the whole egg is graphically represented in Figure 1, A and these values are strikingly similar to those reported earlier by Hill (1945). An examination of this curve shows a marked initial increase during pre-diapause, which is probably due to the formation of carbohydrate-containing cuticles. This increase is followed by a drop in total carbohydrate to a value which remains rather constant throughout the remainder of embryonic development. The general significance of the carbohydrate content of the whole egg for development has been discussed by Hill (1945) and no further reference to it will be made at this time.

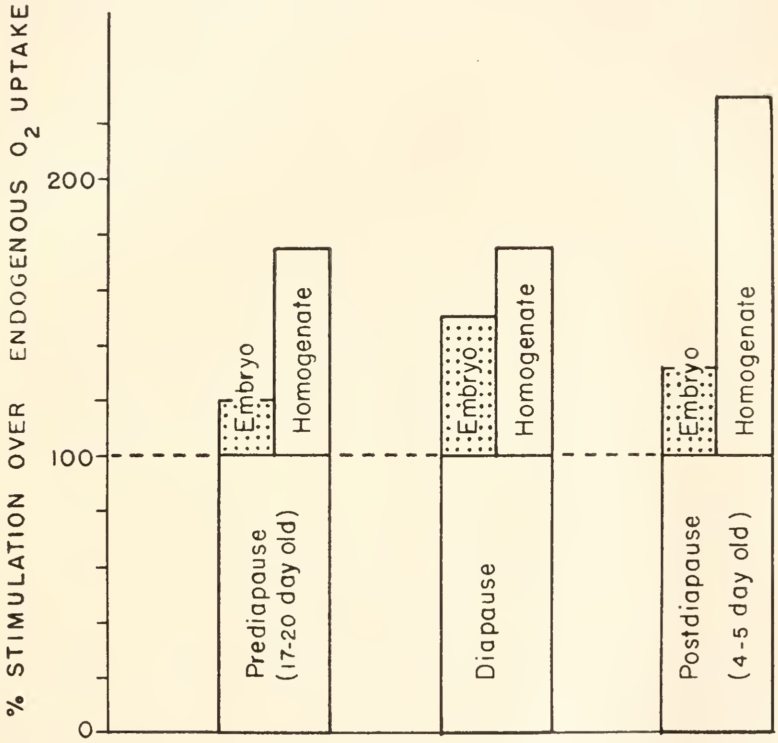


FIGURE 4. Shows percentage stimulation over the endogenous $O_2$ uptake of intact embryos and homogenates due to addition of substrates, glucose-1-$PO_4$, fructose-6-$PO_4$, in final concentration of 0.026 *M*. Stages of development indicated on abscissa; per cent stimulation on ordinate. Data taken from averages of all experiments.

The concentration of carbohydrates in the yolk is graphically presented in Figure 1, B. An examination of this curve shows that from the time of laying to about the fifth day pre-diapause, there appears to be an increase of approximately

19 $\mu$gms. of carbohydrate per egg. This rise is followed by a fall of approximately 40 $\mu$gms. carbohydrate per egg between the fifth and fifteenth days of pre-diapause development. This latter concentration is maintained throughout the remaining pre-diapause and diapause developmental periods but falls again during post-diapause (Fig. 1, B).

The concentration of the TCA-soluble carbohydrate of the yolk (Fig. 1, D) is considered to represent the hexoses. These concentrations are similar to the free reducing substances of Hill, who also suggested them to be hexoses. An analysis of curve D reveals a drop of approximately 32 $\mu$gm. carbohydrate per egg from 15-day pre-diapause to diapause. A constant level is noted during diapause and a rise of 20 $\mu$gms. carbohydrate per egg by 10-day post-diapause development. The TCA-soluble carbohydrate (hexose) content of the embryo (Fig. 1, C) is higher in both pre-diapause and post-diapause than in diapause. This result agrees qualitatively with that presented by Hill (1945). The values recorded here, however, are approximately one-tenth of those presented by him for the free reducing substances of the embryo.

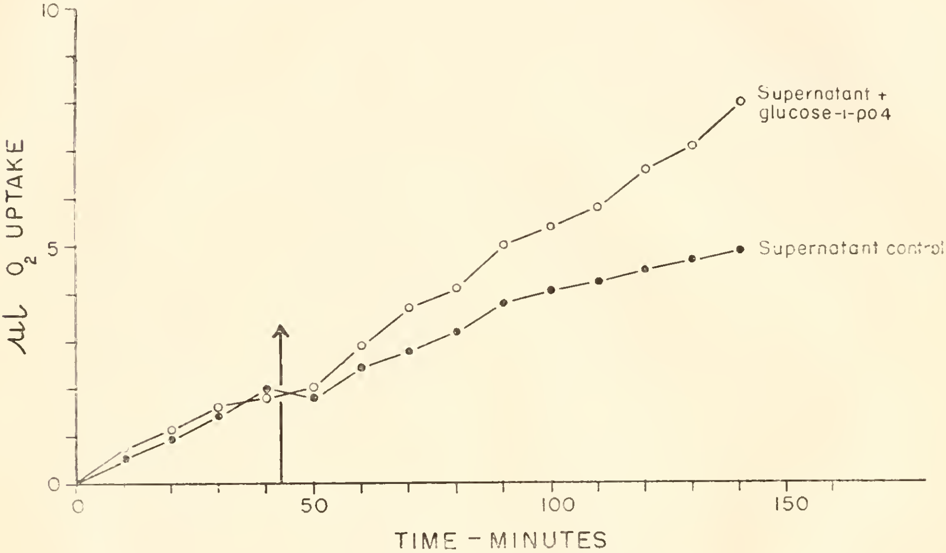


FIGURE 5. Same as Figure 2 except for cytoplasm of diapause embryo.

## RESPIRATION OF THE ISOLATED, INTACT EMBRYO

Embryonic respiration during the mitotically active and blocked stages of development is stimulated by the addition of hexose monophosphates while glucose has no effect over a 100-minute exposure (Figs. 2 and 4). The percentage stimulation due to the hexose phosphates during diapause or blocked periods appears greater than that in pre- and post-diapause (Fig. 4). Hexose diphosphate, although not extensively investigated, seems to act similarly to hexose monophosphates.

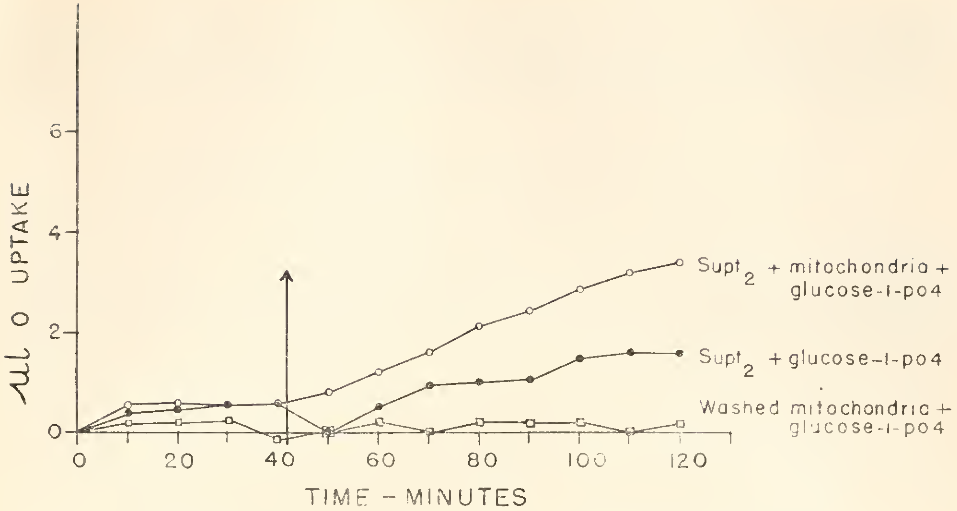


Figure 6. Same as Figure 2 except for fractionated cytoplasm.

## Homogenate Respiration

The endogenous respiration of broken embryonic cells (homogenate) is markedly stimulated by hexose monophosphates in the presence of $Mg^{++}$ (Fig. 3). $Mg^{++}$ alone does not affect the endogenous respiration of homogenates (Fig. 3).

Hexose diphosphate shows a much lower percentage increase of stimulation of

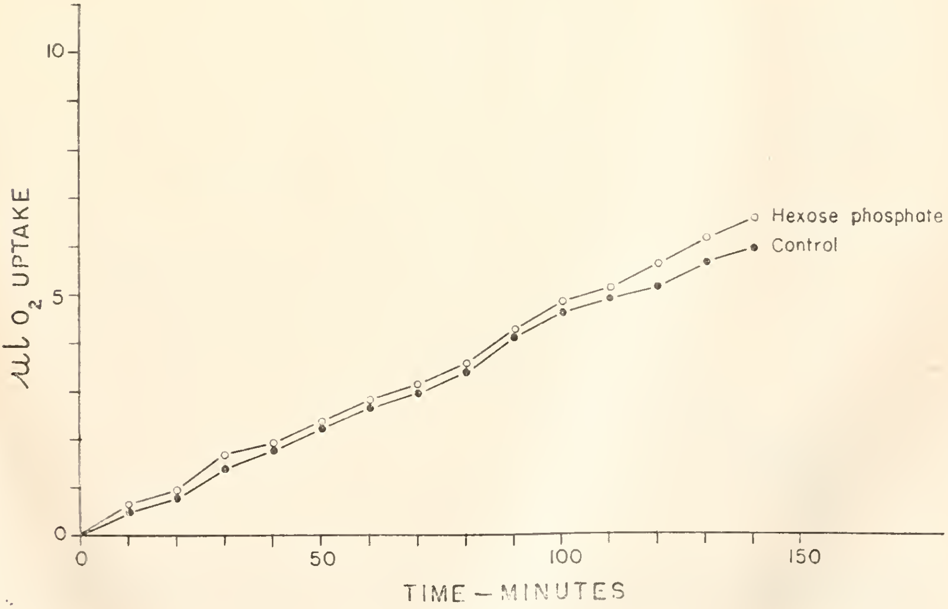


Figure 7. Same as Figure 2 except for isolated nuclei.

endogenous respiration than the hexose monophosphates (Fig. 3). This weak aldolase activity was unexpected and will be dealt with in a later paper.

The percentage stimulation of endogenous respiration tends to increase during post-diapause development (Fig. 4).

## Fractionation and Inhibition of Cellular Respiration

As previously indicated, the supernatant or cytoplasmic fractions of the centrifuged embryo homogenates seem to contain the aerobic glycolytic enzymes (Fig. 5). When the mitochondria are removed from the supernatant the aerobic glycolytic functions are slightly reduced (Fig. 6) while the washed mitochondria show no glycolytic action whatever (Fig. 6). Mitochondria added to supernatant from

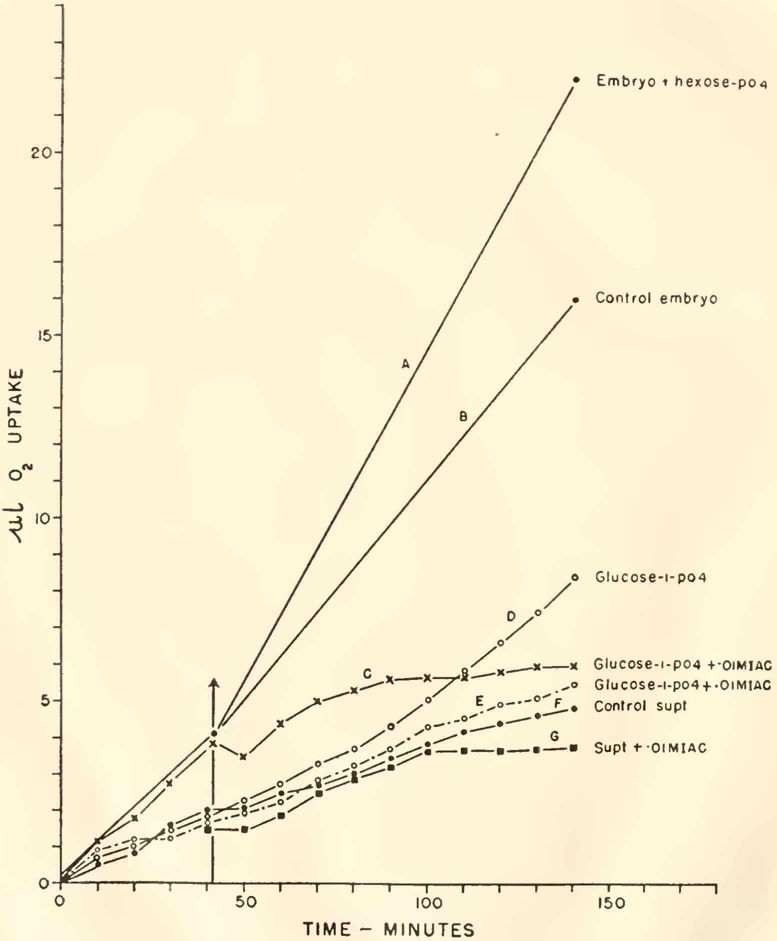


Figure 8. Shows effect of iodoacetate (IAC) on $O_2$ uptake of intact diapause embryo and isolated cytoplasm. A, B, C intact embryo; D, E, F, G cytoplasm. Otherwise same as Figure 2.

which they were removed tend to show an added action over the supernatant alone. Once the original fraction is broken down into its various constituents and these in turn again added together, the glycolytic action (aerobic) is greatly reduced over that of the non-fractionated supernatant. No aerobic glycolytic action occurs in the isolated nuclei (Fig. 7).

Iodo-acetate (0.01 $M$) markedly inhibits the aerobic metabolism of hexose phosphate both in the intact embryo and its cytoplasm (Fig. 8).

## Discussion

Some chemical changes in carbohydrates during development of the egg and embryo of the grasshopper have been previously investigated in this laboratory, using the Hagedorn-Jensen method for reducing sugars (Hill, 1945). Hill discussed fully the changes in carbohydrates in relation to extra-embryonic membrane formation, embryonic development and energetics. He suggested that the chemical changes in the egg are consistent with Needham's theory of a definite sequence in which metabolites are used and also with the concept that terrestrial eggs during development burn fat predominantly.

It is apparent from the present results that an initial fall in carbohydrate content of the yolk between 0-day and 15-day pre-diapause is followed by no marked change in the yolk until the post-diapause period. The curve for total carbohydrates (Fig. 1, A) also reveals qualitatively similar results. Hill associates these early (pre-diapause) and late (post-diapause) changes with the carbohydrate phase of metabolism and extra-embryonic membrane formation which probably involves protein metabolism, and also suggests that the increased carbohydrates may be in part supplied by conversions of protein.

The carbohydrate lost by the yolk in post-diapause is apparently gained by the embryo since a constant carbohydrate content in the acid hydrolysis fraction exists (Fig. 1, D).

A comparison of the oxygen consumed during the stages 18–20-day pre-diapause, diapause and 4–5-day post-diapause shows that changes in carbohydrate content cannot account for the increased intensity of aerobic metabolism of the intact egg (Hill, 1945). However, the loss in hexoses (10% TCH-soluble carbohydrate) of the embryo and yolk during pre-diapause is more than sufficient to account for the increased respiratory activity of the intact embryo. Thus, carbohydrate may act as a source of energy for the marked synthesis within the embryo. This is further supported by data on the R.Q. which is slightly higher for the isolated embryo than for the intact egg.

Bodine and Boell (1934) observed that mitotically active post-diapause embryonic cells can utilize glucose, a stimulation in $O_2$ uptake occurring after an exposure of two hours. The results presented here for 100 minutes exposure do show that the embryo can utilize hexose phosphates to a greater extent than glucose over the same length of time (Figs. 2 and 4). Phosphorylated hexose seems to be utilized whether the cells are mitotically active or blocked, intact or broken. (The $Mg^{++}$ ion is necessary for utilization by broken cells.) This suggests that hexokinase activity is either absent or present in very small amounts as compared with the enzymes necessary for the aerobic breakdown of these phosphorylated intermediates.

The apparent absence of hexokinase is paralleled by a lack of alkaline phosphatase activity in the embryo until approximately 8 days post-diapause. Fitzgerald (1949) suggested that alkaline phosphatase might be concerned in the transport of food material to the embryo, since consistent increase in phosphate in both pre- and post-diapause is paralleled by an increased alkaline phosphatase activity of the yolk.

The free reducing substances of Hill and the present TCH-soluble carbohydrate (Fig. 1) are a possible source of hexose, with phosphorylation perhaps occurring within the yolk itself or at the cell membrane of the embryo.

The percentage stimulation of embryonic respiration using hexose monophosphates as substrate varies markedly over the stages investigated (Fig. 4). It is greatest in diapause. However, the percentage stimulation of the homogenate does not parallel that of the embryo either qualitatively or quantitatively, being greatest in mitotically active post-diapause. This may indicate that diffusion is a limiting factor, particularly since the concentration of TCA-soluble carbohydrate is higher in 17–20-day pre-diapause and 4–5-day post-diapause than that in diapause. Further, the maximal increase in homogenate stimulation is similar for both diapause (blocked) and pre-diapause (active) while the embryonic stimulation varies, indicating perhaps a higher relative saturation of the enzyme in the intact mitotically active embryos. Moreover, the constancy of stimulation of the homogenate in pre-diapause and diapause indicates little, if any, synthesis of enzymes associated with aerobic glycolysis during this developmental period. Either a marked synthesis or increase in activity must occur at post-diapause, as shown by the great increase in stimulation of the homogenate.

Following separation of the embryo homogenate into supernatant and nuclear fractions, the enzymatic activity was found to reside primarily in the supernatant. The mitochondria (large granules) isolated from the supernatant by centrifugation were not stimulated by added hexose phosphates. The supernatant (microsomes and remaining cytoplasm) shows very weak stimulation, while a combination of the two fractions gives a higher percentage $O_2$ uptake but not equivalent to the original supernatant itself. Thus, the enzymes associated with aerobic glycolysis appear more intimately associated with the soluble protein fraction, as has been shown to be the case for mammalian material.

Iodoacetate, a dehydrogenase inhibitor, believed by some to be specific for aldolase activity, inhibits almost completely the stimulation of the hexose monophosphates. A recent article by Cleland and Rothschild on the oxidation of carbohydrate of the sea-urchin egg strongly suggests metabolic changes in this material similar to those for the grasshopper embryo (Cleland and Rothschild, 1952).

## SUMMARY

1. A study has been made of the carbohydrate content and changes in the intact egg and isolated embryo of the grasshopper, *Melanoplus differentialis*, during the course of embryonic development.

2. Changes in the carbohydrate content of yolk have been compared with similar changes in the developing embryo.

3. Respiration of intact isolated embryos in different carbohydrate substrates indicates a marked utilization of phosphorylated compounds.

4. The endogenous oxygen uptake of embryo homogenates is markedly stimulated by hexose monophosphates in the presence of $Mg^{++}$.

5. Aerobic glycolysis seems to be controlled by enzymes located only in the cytoplasm of the embryonic cells.

6. Phosphorylation of carbohydrates occurs either in the yolk or at the surface of the embryonic cells.

## LITERATURE CITED

Bodine, J. H., and E. J. Boell, 1934. Respiratory mechanisms of normally developing and blocked embryonic cells. *J. Cell. Comp. Physiol.,* **5**: 97–113.

Bodine, J. H., and E. J. Boell, 1936. Respiration of embryo versus egg. *J. Cell. Comp. Physiol.,* **8**: 357–366.

Bodine, J. H., and K. Lu, 1950a. Oxygen uptake of intact embryos, their homogenates and intracellular constituents. *Physiol. Zool.,* **23**: 301–309.

Bodine, J. H., and K. Lu, 1950b. Methylene blue, 2,4-dinitrophenol and oxygen uptake of intact and homogenized embryos. *Proc. Soc. Exp. Biol. Med.,* **74**: 448–450.

Bodine, J. H., and K. Lu, 1951. Structure and endogenous oxygen uptake of embryonic cells. *Physiol. Zool.,* **24**: 120–126.

Bodine, J. H., K. Lu and W. L. West, 1952. Reduction of triphenyltetrazolium chloride by mitotically active and blocked embryonic cells. *Biol. Bull.,* **102**: 16–21.

Boell, E. J., 1935. Respiratory quotients during embryonic development. *J. Cell. Comp. Physiol.,* **6**: 369–385.

Cleland, K. W., and Lord Rothschild, 1952. The metabolism of the sea-urchin egg. Oxidation of carbohydrate. *J. Exp. Biol.,* **29**: 416–428.

Dreywood, Roman, 1946. Qualitative test for carbohydrate. *Ind. Eng. Chem. Anal. Ed.,* **18**: 499.

Fitzgerald, L. R., 1949. The alkaline phosphatase of the developing grasshopper egg. *J. Exp. Zool.,* **110**: 461–487.

Hill, D. L., 1945. Carbohydrate metabolism during embryonic development (Orthoptera). *J. Cell. Comp. Physiol.,* **25**: 205–216.

Morris, D. L., 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science,* **107**: 254–255.

Morse, E. E., 1947. Anthrone in estimating low concentrations of sucrose. *Ind. Eng. Chem. Anal. Ed.,* **19**: 1012–1013.

Needham, J., 1942. Biochemistry and morphogenesis. Cambridge University Press, Cambridge, England.

Spratt, N. T., Jr., 1952. Metabolism of the early embryo. *Ann. N. Y. Acad. Sci.,* **55**: 40–49.

Viles, Frederick J., Jr., and Leslie Silverman, 1949. Determination of starch and cellulose with anthrone. *Ind. Eng. Chem. Anal. Ed.,* **21**: 950–953.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

# INDICATOR GRADIENT PATTERNS IN OÖCYTES AND EARLY DEVELOPMENTAL STAGES OF ECHINODERMS: A REEXAMINATION

C. M. CHILD

*School of Biological Sciences, Stanford University, Stanford, Calif., and Hopkins Marine Laboratory, Pacific Grove, Calif.*

The gradient patterns, as observed by means of intracellular reduction of methylene blue and diazine green (Janus green), following staining by oxidized dyes were recorded for cleavage and later stages of the echinoids, *Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus* and for the asteroid starfish, *Patiria miniata,* and for exogastrulae of these forms in earlier papers (Child, 1936a, 1936b). Later the pattern of the intracellular indophenol reaction during larval development of *Dendraster* was determined, together with some data on patterns of exogastrulae (Child, 1941). In these observations on indophenol pattern, extremely low concentrations of reagents were used so that the patterns became visible in living animals. Still later, data concerning the indophenol gradient patterns of *Patiria miniata* from the later ovarian oöcytes to larval stages, also with very low concentrations of reagents, appeared (Child, 1944).

However, intracellular oxidation of reduced dyes and of indophenol reagents has been found more satisfactory than reduction after staining in the study of gradient patterns, for reasons noted in the following section. The present paper is primarily concerned with new data concerning gradient pattern, as determined by intracellular oxidation of indicators in stages from early ovarian oöcytes through embryonic development to larval stages of certain echinoderms.

## MATERIAL AND METHODS

Two echinoids, *Strongylocentrotus purpuratus* and *Dendraster excentricus,* and the asteroid starfish, *Patiria miniata,* have served as material. The data include stages from the early ovarian oöcytes to earlier larvae of the echinoids. As regards the starfish, they are merely supplementary to the earlier study of the indophenol reaction in this form (Child, 1944). For the two echinoids these new data on intracellular oxidation of indicators constitute a more complete survey of indicator patterns than earlier studies and render differentials more clearly visible, particularly in certain stages. Deep indebtedness to the Director and staff of the Hopkins Marine Laboratory of Pacific Grove for collecting material, providing facilities for its use and, in various cases, for transporting it to Palo Alto, is gratefully acknowledged. For fertilization and development at Palo Alto filtered sea water kept in darkness has been found satisfactory.

In the first studies of indicator patterns in early echinoderm development (Child, 1936a, 1936b), the material was stained by an oxidized redox dye and reduction was brought about by oxygen uptake of embryos and larvae sealed in a small volume of water or, in some cases, of dye solution. The decrease in free oxygen content of the solution brought about intracellular reduction of the dye. Reduction under these conditions was very slow and uniform distribution of oxygen was provided for by frequent change of position of the sealed material in relation to gravity or with motile stages by swimming activities. Reducing agents which had been used in earlier indicator studies were highly toxic and might themselves retard or inhibit reduction differentially by their toxicity.

As indicator studies continued it became increasingly evident that intracellular oxidized dyes, with increase in intracellular concentration, might also retard or even inhibit intracellular reduction differentially. This retardation or inhibition occurred most rapidly in the regions which reduced most rapidly in uninjured material; *i.e.,* if the oxidized indicator attained a certain intracellular concentration it might decrease or even obliterate slight regional differentials of pattern in consequence of the differential susceptibility of different gradient levels to the dyes, particularly the more toxic dyes such as diazine green (Janus green). In some cases a gradient pattern has become reversed in direction by the differential retardation or inhibition of intracellular reduction. In use of reduced dye solutions for intracellular oxidation, the toxic reducing agents might alter gradient pattern before intracellular oxidation began.

These difficulties are almost entirely avoided by use of sodium hydrosulphite as a reducing agent, either for dye solutions to be used for intracellular oxidation or for intracellular reduction after staining by an oxidized dye. This agent is not appreciably toxic, even with hours of exposure to concentrations far above those required to reduce the indicators, either in solution externally or intracellular. Very small quantities of this agent reduce dye solutions in a few seconds and it can be used at once for intracellular oxidation or for intracellular reduction of material previously stained by an oxidized dye. In the present paper it is used primarily for intracellular oxidation of dyes. A few minute granules of hydrosulphite, less than a milligram, are sufficient to reduce completely one ml. of methylene blue or diazine green solution and various other dyes in concentrations found useful. Intracellular oxidation occurs within a few minutes without evidence of toxic effect until intracellular dye concentration becomes high. Care must be taken not to use an excess of hydrosulphite beyond that required to reduce the dye. Any considerable excess may retard or even prevent intracellular oxidation in consequence of too complete removal of oxygen. After intracellular oxidation addition of a little more hydrosulphite will result in differential intracellular reduction. In many organisms differential oxidation and reduction can be repeated without alteration of pattern. These methods of using sodium hydrosulphite, although noted in earlier papers, are described in detail here because their use on early stages of echinoderm development is regarded as a somewhat critical case near the lower limit of availability of redox dyes for rendering visible regional differentials of pattern. Intracellular oxidation from reduced dye solutions has been called for convenience primary oxidation in order to distinguish it from re-oxidation, following reduction.

Redox dyes used for intracellular oxidation, often with following reduction, were methylene blue in various concentrations from 1/50,000 to 1/20,000, diazine green from 1/100,000 to 1/50,000 and Nile blue sulphate 1/100,000. These were used chiefly on *S. purpuratus* for comparison with the indophenol reaction; on *Dendraster* and *Patiria* the indophenol reaction was used almost entirely. Primary oxidation of the redox dyes and the intracellular oxidation of the indophenol reagents have made visible the same patterns, though in certain cases the indophenol reaction has shown the differential more distinctly than the dyes.

The indophenol or Nadi reaction, the intracellular formation of the deep blue indophenol from para-aminodimethylaniline (dimethyl-paraphenylene diamine) and α-naphthol, catalyzed by an oxidase, "indophenol oxidase," commonly regarded as cytochrome oxidase or a related enzyme, has been found extremely useful in the studies of echinoderm pattern and in many other organisms. In several earlier papers the modifications of the reaction found most useful in rendering visible the slight differentials of early developmental stages and small single cells have been described. However, since presence of a gradient pattern in early stages of echinoderm development has been questiond, it has seemed necessary to call attention once more in some detail to the method, as used with low concentrations of reagents. Also certain points not particularly considered earlier are noted.

The aniline has been obtained in 10 gram amounts from the Eastman Kodak Co. Some of these have been liquid, others solid at room temperature and, according to information from the Kodak Co. laboratories, the solid form is more nearly pure. However, the melting point is only slightly above room temperature and immersion of the bottle containing the aniline in slightly warmed water liquefies it. Since the aniline is volatile and an acrid, highly irritating poison, weighing of the very small amounts required presents difficulties, and since earlier use of the aniline was in terms of small drops of the liquid, its use has been continued in this way. Although attempts have been made in earlier papers to give information concerning concentrations used, the actual concentrations are not known in any particular case and since the same pattern of reaction appears with a very wide range of concentrations and the method is not quantitative, the chief point at present is use of concentrations found by experience to give the reaction with as little indication of toxicity as possible. For most organisms and even for many single cells a solution, consisting of one small drop (30–40 = 1 ml.) in 10 ml. of salt or fresh water, according to the material, is made daily or oftener; the aniline gradually oxidizes in water. The concentration of this solution can be altered as desired. This is essentially a stock solution and is diluted for use. The α-naphthol stock solution consists of one mg. or less, estimated after repeated weighings as a basis for estimation, in 10 ml. of water, salt or fresh. Enough naphthol dissolves within a few minutes to give the reaction readily without addition of KOH or NaOH necessary to dissolve the naphthol when high concentrations of the agents are used.

The solution for use, regarded as standard, merely because it provides a starting point for determining the most satisfactory solution for a particular material, consists of one drop each of the aniline and the naphthol stock solutions in one ml. of water. The naphthol is much less toxic than the aniline and can be used in considerably higher concentrations if desired, but in any case it is not certain just how much of the naphthol dissolves without addition of alkali. In some organisms and with some cells concentrations half or a fourth of the standard, or even less, are desirable. Solutions four to five times the standard are usually rather rapidly toxic. In general the practice found most effective has been to use the lowest concentrations of reagents which render the intracellular reaction clearly visible within a reasonable period of observation, *i.e.*, 10–30 minutes, although it is often possible to obtain a very distinct differential reaction in four to five minutes. The method is of course at present far from quantitative; its use in any case is a matter of trial and error, but it does render directly visible certain characteristics of physiological pattern in living organisms which are not now directly distinguishable in any other way, except with the method described above with redox dyes. With these general ranges of concentration and variations from them as occasion required, the indophenol reaction has made directly visible patterns of oxidase activity in numerous organisms from protozoa to vertebrates. At least the earlier stages of the reaction occur in apparently uninjured individuals; motile forms continue activity and non-motile developmental stages or forms may continue development until the intracellular concentration becomes high. With the much higher concentrations of the reagents, about 0.1 per cent and in some cases even one per cent, in some of the earlier work with the reaction and with alkali added to dissolve the α-naphthol, the material must have been killed almost at once, and little or no gradient pattern remained. For the echinoderm material twice the standard was commonly used. somewhat less often, the standard solution.

Some organisms apparently reduce or otherwise destroy intracellular indophenol with partial or complete loss of color, after the reaction has continued for a time, perhaps because of stimulation or irritation by the intracellular indophenol or the aniline. This decrease or loss of color has sometimes been observed in later echinoid blastulae and in gastrulae. Also, unless the intracellular concentration is very high there is usually more or less reduction with loss of color when cytolysis occurs. Intracellular reduction occurs rapidly on addition of very small amounts of sodium hydrosulphite, provided the intracellular concentration of indophenol has not become so high that reduction is retarded or inhibited. Re-oxidation and perhaps a second reduction are often possible without alteration of pattern.

The patterns of primary intracellular oxidation from dye solutions reduced by sodium hydrosulphite and of indophenol are similar but the indophenol reaction seems to show very slight differentials in the echinoderm material a little more distinctly than dye oxidation. Reduction patterns of the dyes and of indophenol are also similar. It is perhaps unnecessary to point out that the earlier stages of the indophenol reaction and also of primary dye oxidation are more important than the later stages in making visible the regional differentials of pattern. With increasing intracellular concentrations of oxidized dyes and of indophenol, slight differentials become less distinct or disappear as the color approaches uniformity. With low concentrations of dyes and indophenol reagents these methods are an exceedingly delicate means for showing directly slight regional differentials in activity of an oxidase or of oxidases and in reduction, of one or more dehydrogenases. They have an advantage over various other methods of not requiring separation of the organism into pieces.

In observation of these indicator patterns the most extreme precautions have been taken to avoid being deceived by apparent differentials resulting from direction or character of illumination or other extraneous factors. Frequent agitation has provided for uniform concentrations of dyes and indophenol reagents in non-motile stages. In motile stages the swimming activity of the animals serves this purpose. Single eggs have been moved about and turned over repeatedly. The patterns noted in this paper have been seen in hundreds of individuals during earlier and later parts of the breeding seasons and in different lots of material during the last five years.

Figures are diagrammatic outlines with the gradients indicated by differential shading or by arrows pointing in the general direction of decrease in rate of intracellular oxidation, *i.e.*, down the gradient. Early cleavages of *S. purpuratus* and of *Dendraster excentricus* are alike. *Dendraster* eggs and cleavage stages are larger than those of the sea urchin but separate figures for these stages of the two species are regarded as unnecessary. The egg and developmental stages of the starfish, *Patiria,* are much larger than those of the echinoids and are outlined in the figures as slightly larger, but do not indicate the actual differences in size. In the shaded figures the shading is an attempt to indicate the differential as observed in particular cells, and nuclear position in the cell is drawn as observed. Arrows indicate merely the general directions of gradients, not their extent. Shorter arrows indicate less differential.

## GROWTH STAGES OF OVARIAN OÖCYTES

In the earlier study of intracellular reduction of redox dyes in the ovarian oöcytes of *S. purpuratus, S. franciscanus* and *Patiria miniata,* rate of reduction usually decreased from that region of the cell where the nucleus was nearest the surface (Child, 1936a; Figs. 1–3, 8 and 9). This region varied with respect to attachment to ovarian tissues but was usually somewhere on the surface not in contact with ovarian tissues, though a few very early oöcytes were observed with reduction gradient decreasing from the attached pole and nucleus near that pole (Child, 1936a; Fig. 4). These may have been stages so early in oöcyte growth that other ovarian tissues still reduced more rapidly than the oöcyte. Figure 5 of the same paper with reduction decreasing in rate from the interior of the cell after staining by oxidized diazine green, is probably a case of differential injury with retardation of reduction near the cell surface; with intracellular oxidation nothing similar has ever been observed.

It is usually possible to find early oöcyte stages by gentle teasing of small portions of the ovaries, even in females with large numbers of eggs ready for fertilization. Figure 1, *a–x*, the result of repeated series of observations on all three echinoderms constituting the material of this paper, is believed to be a fairly adequate sample of the gradient patterns observed in the earlier stages of oöcyte growth and of the nuclear positions in relation to these patterns. In Figure 1, *a–l* are from *S. purpuratus, m–q* from *Dendraster* and *r–x* from *Patiria.* In most of these cells the relation to the ovarian tissue is not evident. In those which do show some indication of this relation (Fig. 1, *f, k, n, o*) the region of most rapid intracellular oxidation is at or near the "free" pole of the cell. The oöcytes differ in form and are often more or less elongated, the axis of elongation being the gradient axis or departing only slightly from it. The gradient pattern in these cells is distinct, though with apparent variation in differential in different cells. In most cases the rate of oxidation decreases rapidly from one pole with relatively slight differential in the remainder, often a half of the cell. This condition has seemed to be more distinct in *S. purpuratus* and *Dendraster* than in *Patiria,* but whether it is of real significance remains uncertain. It is apparently more frequent in the earlier stages and may perhaps indicate that the gradient is in process of developing from the high end.

Nuclear position in relation to the gradient pattern varies. In the earliest stages (Fig. 1, *a–d, r*) it may be at or near the pole of least rapid reaction, the "low" end of the gradient or in some other region of the cell, perhaps near the middle (*g, j, k, s, w*) and not always in the gradient axis. However, in the great majority of the oöcytes the nucleus is near the high end of the oxidation gradient and apparently more frequently in this position as size of the oöcyte increases, but a cell approaching full size has occasionally been seen with nucleus near the middle. These observed differences may be without real significance, but if they do represent actual physiological differences in individual cells they suggest that gradient pattern is developing in the oöcytes, that different stages of this development appear in different cells and that the nucleus gradually comes to lie near the high end of the gradient, though some variation in position may still occur in later stages.

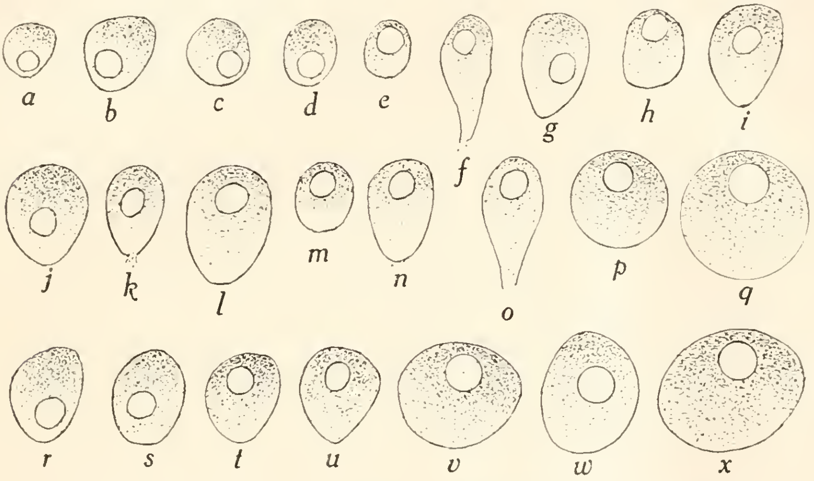


FIGURE 1. Gradient patterns of early oöcytes: *a–l, S. purpuratus; m–q, Dendraster excentricus; r–x, Patiria miniata.*

As the cell increases in size the gradient differential seems to become less distinct, *i.e.*, the lower gradient levels differ less from the higher levels than in many earlier stages, and in general the gradient appears to decrease in rate of reaction, probably as enzyme activity decreases in the later stages of growth. However, it is difficult to determine whether changes such as this are or are not significant. It is certain that the oxidation gradient pattern does become less distinct in the full grown unfertilized egg than in the early oöcyte stages.

All oöcytes of Figure 1 are placed in the figure with the region of most rapid oxidation uppermost. This involves the assumption that the gradient pattern represents a definite physiological axis, rather than a mere chance differential differing from cell to cell and without further significance. Moreover, even though these early stages alone do not provide evidence that the high ends of their gradient patterns become the apical or animal pole of the egg, comparison with later stages leaves little doubt that this is the case. It appears highly improbable that a gradient pattern involving enzymes of fundamental importance is without definite relation to oxidation patterns of later stages. It is believed, therefore, that these data concerning early stages in ovarian egg development indicate a developing apicobasal physiological axis that persists and becomes the polarity of the egg and embryo.

With further growth of the oöcyte the relation of nuclear position to the high end of the indicator gradient becomes increasingly definite, though some variation still occurs. The usual nuclear position in later growth stages is indicated in Figure 2, but even in these stages an oöcyte with nucleus near the center of the cell has occasionally been seen. These cells are perhaps not in good condition. With progress of growth the indophenol reaction becomes much less rapid and the gradient differential is slight but still distinguishable with care in use of the reaction and in observation. As it approaches the end of the growth period the cell evidently becomes less active, at least so far as the oxidase or oxidases catalyzing the reaction are concerned, and preceding fertilization, or, in the case of the starfish, preceding polar body formation, it is an extremely inactive cell. In many of these cells, however, a slight gradient is still visible; in some others it has not been distinguished with certainty.

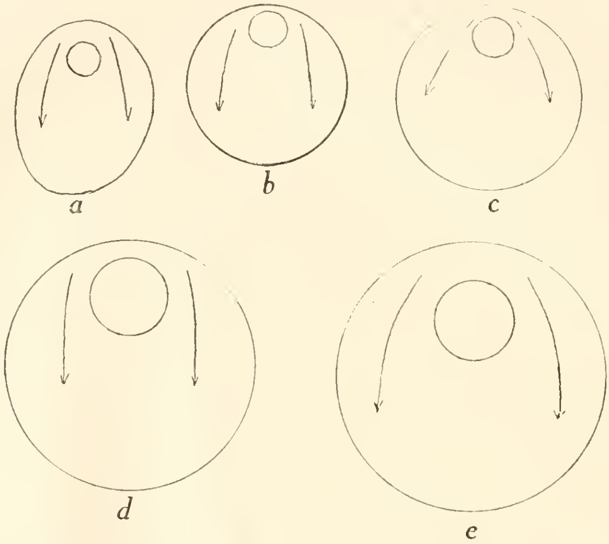


Figure 2. Gradient patterns of later growth stages of oöcytes: *a–d, S. purpuratus* and *Dendraster; e, Patiria.*

## Polar Body Stages

In the two echinoids the first polar body is formed in the ovary preceding spawning. In slightly teased ovaries it has been seen occasionally (Fig. 3, *a, b*). It is formed at the high end of the slight gradient. It has sometimes seemed that the gradient became slightly more distinct at the time of its formation. There is every reason to believe that the pole near which the nucleus lies in Figure 2 and the region of polar body formation are the same. It appears highly improbable that the nucleus has moved to another region of the egg after the growth period of the oöcyte has ended. The nucleus of course becomes invisible at the time of polar body formation and afterward it is smaller but can usually be made visible by pressure (Child, 1936a; Fig. 7). The second polar bodies of the two echinoids are formed after fertilization (Fig. 3, *c, d*). The intracellular indophenol reaction becomes more rapid and the gradient more distinct following fertilization. The *Patiria* eggs were fertilized as soon as possible after extrusion from the gonads and both polar bodies formed after fertilization (Fig. 3, *e*), also with increase in distinctness of the indophenol gradient. If the *Patiria* eggs had been kept without fertilization, polar body formation and activation would probably have occurred as in *Asterias* (Loeb and Wasteneys, 1912; Tang, 1931). Polar bodies formed at the pole of most rapid indophenol reaction.

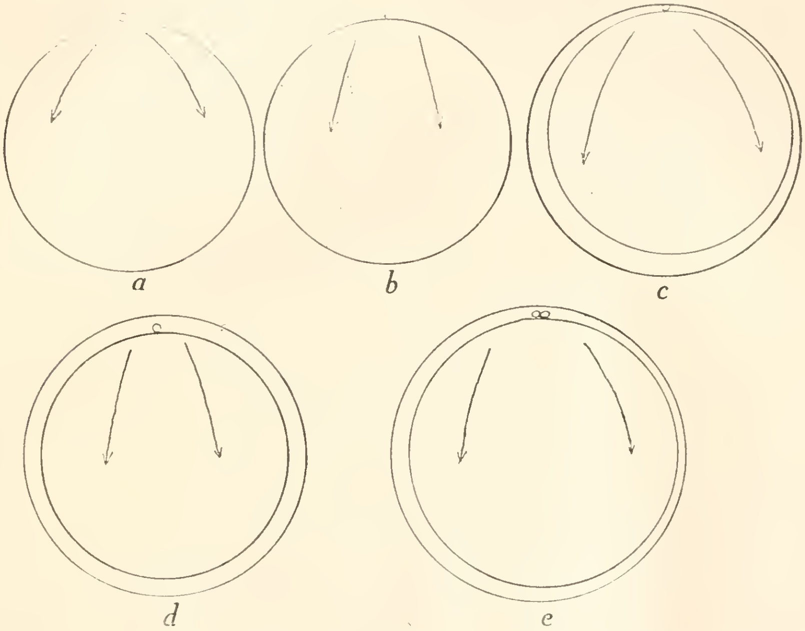


Figure 3. Gradient patterns of polar body stages: *a* and *b*, first polar body stage of *S. purpuratus* and *Dendraster* before fertilization; *c* and *d*, second polar body stage of these forms following fertilization; *e*, polar bodies of *Patiria*, following fertilization.

## Early Cleavage Stages

The gradient pattern in early cleavage stages of *S. purpuratus* has been rendered visible by primary intracellular oxidation of reduced methylene blue, diazine green and Nile blue sulphate as well as by the indophenol reaction, the same pattern appearing with all three procedures. Only the indophenol reaction has been used on most lots of *Dendraster* and *Patiria* with the same results in all cases. The observations on these stages have been made repeatedly on many lots of eggs during different breeding seasons from 1947 on. They have included thousands

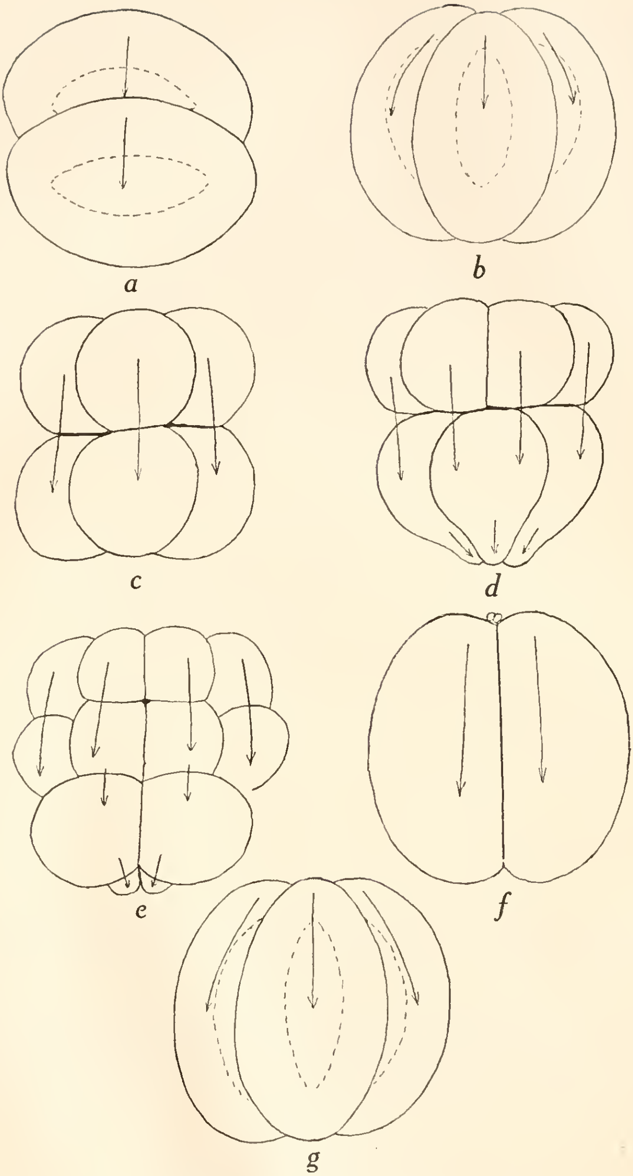


Figure 4. Gradient patterns of early cleavage stages: *a* and *b*, 2- and 4-cell stages of *Strongylocentrotus* and *Dendraster* with outlines of mitotic figures for next division; *c* and *d*, 8- and 16-cell stages of the two echinoids, difference between mesomeres and macromeres distinct, between macromeres and forming micromeres less distinct but visible; *e*, 24-cell stage of the echinoids, difference between mesomeres and macromeres apparently slightly more distinct in many embryos, in others essentially like 16-cell stage, micromeres still slower reaction than macromeres; *f* and *g*, 2- and 4-cell stages of *Patiria*.

of eggs under widely varied conditions of illumination. Early cleavages of *S. purpuratus* and *Dendraster* are alike, and in Figure 4, *a–e* indicate gradient pattern in both. In these forms the second polar body usually lies deep in the cleavage furrow and is often not certainly visible. For certain orientation, in Figure 4, *a,* the 2-cell stage is drawn with outlines of the mitotic figures for the second cleavage indicated. In Figure 4, *b,* the 4-cell stage, mitotic figure outlines for the third cleavage are also indicated. These are slightly nearer the pole of most rapid dye oxidation and indophenol reaction and the four apical cells of the 8-cell stage are usually slightly smaller than the basal cells (Fig. 4, *c*), though there is apparently some variation in this respect. In the 8-cell stage the differential between the apical and basal cells becomes even more distinct. In Figure 4, *d,* approaching 16 cells, the differential from mesomeres to macromeres is clearly evident and the forming micromeres show even less rapid reaction than the macromeres. At the stage of Figure 4, *d,* dye oxidation and indophenol reaction are more rapid in the apical than in the basal mesomeres with less difference from the basal mesomeres to the macromeres and the micromeres are slowest of all. Figure 4, *f* and *g,* 2- and 4-cell stages of *Patiria,* shows the same gradient pattern as the echinoids and the same as observed in an earlier paper (Child, 1944). No micromeres are formed in *Patiria* but there is a slight increase in size of blastomeres basipetally as cleavage progresses and rate of indophenol reaction decreases basipetally from the smaller cells of the apical region, as also shown in the paper of 1944, so that repetition of those data is unnecessary here. With low intracellular concentrations of oxidized dyes and indophenol it has often been possible to reduce and re-oxidize these cleavage stages without altering gradient pattern.

## The Blastula and Later Stages

In the blastula of all three forms the polar gradient pattern becomes so distinct that it is clearly visible in surface view as well as in optical section. A point of interest to be noted is that the reaction at all levels is more rapid on the blastocoelar side of the cell wall, not on the external surface. In Figure 5, *a* (*S. purpuratus* and *Dendraster*) and *b, Patiria,* the arrows in the cell wall indicate the gradient in optical section, the other arrows the gradient in surface view. The differential from the blastocoelar surface outward is indicated only at the apical end by the optical section arrows drawn from the blastocoelar surface. Thus far it has not been possible to distinguish with certainty a ventrodorsal gradient in blastulae. In some blastulae intracellular oxidation seemed to be slightly more rapid on one side but even if this is actually the case it is not certain that the more rapid side is ventral, as it is in later stages, or whether any probable differential is due to some incidental factor. Probably a ventrodorsal pattern is present at this stage and even earlier but it is either too slight to become distinctly visible in intracellular oxidation or may conceivably differ in character from the ventrodorsal gradient of later stages. In Figure 5, *c,* the gastrula of *S. purpuratus* and in *d* the gastrula of *Dendraster* with somewhat longer apicobasal axis are outlined. Polar and ventrodorsal gradients are both clearly visible in the ectoderm. In the invaginating entoderm a new longitudinal gradient has arisen with rate of oxidation decreasing from the tip. In Figure 5, *e,* the pre-pluteus or early pluteus of the echinoids is outlined in somewhat oblique optical section from the side, with the gradient pattern indicated. The ectodermal polar gradient is becoming more distinct as the oral lobe develops from the apical region. The ventrodorsal gradient is also more distinct than in earlier stages, and a new gradient decreasing from the tip of each developing anal arm is now present. In stages *c*, *d* and *e* the ectodermal pattern is sufficiently distinct to be visible in surface view and optical section. The entodermal differential has also increased. As regards the intracellular indophenol pattern of the later stages of *Patiria* it is unnecessary to repeat here the data recorded in the earlier paper (Child, 1944).

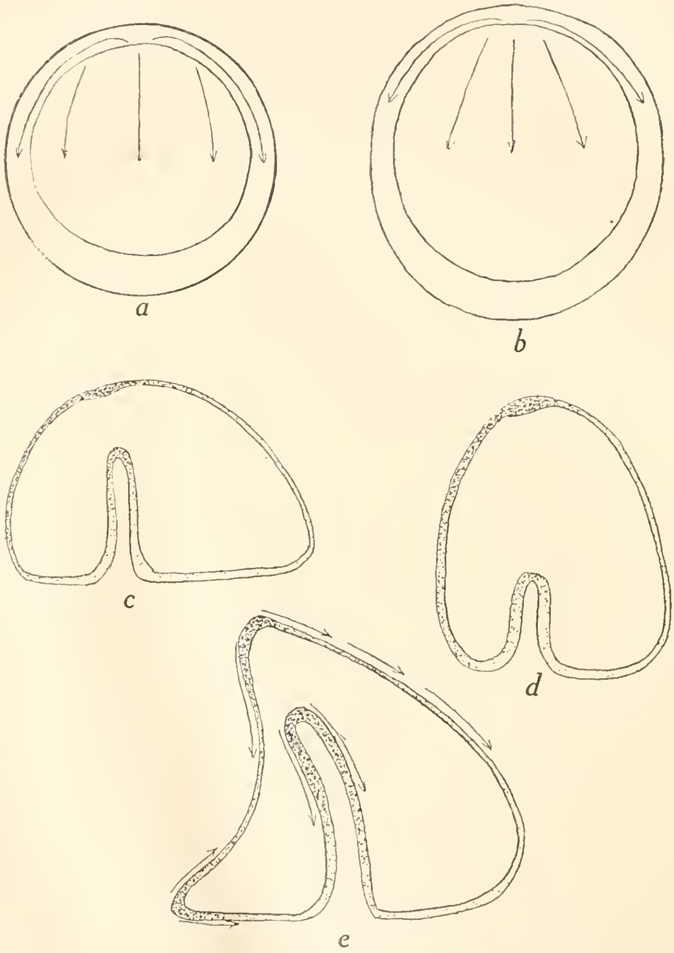


FIGURE 5. Gradient patterns of later stages; differential becomes increasingly distinct during blastula stage. Arrows in cell wall indicate gradient in optical section, other arrows, gradient in surface view. *a*, the two echinoids; *b*, *Patiria; c*, gastrula of *S. purpuratus; d*, gastrula of *Dendraster*, apicobasal, ventrodorsal and entodermal gradients indicated in both; *e*, somewhat oblique outline from side of early pluteus of echinoids, gradient of developing oral lobe in apical region, ventrodorsal gradient, gradient in developing anal arm and gradient in entoderm indicated.

Without feeding, the echinoid plutei gradually die of starvation. During the later stages of their lives intracellular oxidation of reduced dyes and of indophenol reagents becomes less rapid and gradient differentials decrease. Even while they are still to some degree motile they show little evidence of oxidase activity and little or no gradient pattern. Starving larvae of *Patiria* show very similar decrease in gradient pattern and of oxidase activity.

## DISCUSSION

An intracellular oxidation gradient pattern of indophenol reagents and of various reduced redox dyes is present through ovarian development of the oöcyte and through embryonic and larval development. Intracellular oxidation is more effective than reduction in rendering visible slight differentials; staining by oxidized dyes preceding reduction may itself decrease differentials. The differential developmental modifications by external agents show the very definite relations of morphogenesis to this gradient pattern. In the early oöcytes the gradient pattern is very distinct but becomes less so as developmental activity of the oöcyte decreases in later stages. The course of oöcyte development suggests that the cell acquires a cytoplasmic gradient pattern from a differential in ovarian environment, perhaps an oxygen differential, and that the nucleus gradually attains a position near the most active region of this gradient, polar bodies form there and the gradient becomes the basis of physiological polarity in the embryo and larva.

A ventrodorsal pattern of some sort is probably present long before it becomes distinguishable by means of the indicators. It is perhaps of interest to note that in the growth of the ovaries the small finger-like outgrowths show an indicator oxidation gradient decreasing from their tips. Perhaps the oöcyte is subjected from its earliest stages to an ovarian differential at right angles to the polar differential. If this determines a differential in the cell it may be either too slight in early stages to appear, even in intracellular oxidation of indicators, or may not yet have developed a differential in oxidase activity.

The present paper makes it necessary to refer again to the failure of Lindahl and Holter (1940) to find any significant difference in oxygen uptake in apical (animal) and basal (vegetal) halves of developmental stages of *Paracentrotus lividus* and to the somewhat later assertion of Lindahl (1942a, 1942b) that the data of Child concerning reduction gradients in a sea urchin cannot be "valid" because no differential in oxygen uptake was found in the separated halves of embryos. This matter was discussed in an earlier paper (Child, 1944) and only certain points need be noted here. Lindahl's assertion concerning validity seems not entirely justified on the basis of his and Holter's purely negative evidence and in the face of positive evidence to the contrary. These authors do not mention any attempt to determine whether a much larger number of embryo halves than they used would show a differential. Also an error in one of their tables invalidates certain of their conclusions. Moreover, they admit an error of 10 per cent. It has never been maintained that indicator gradients must always parallel regional differentials in oxygen uptake, though they are parallel in various organisms. It is conceivable that separation of the echinoderm stages into parts may decrease or obscure regional differences in oxygen uptake, particularly if these are slight. Possibly also other factors may sometimes be involved in oxygen uptake than those determining indicator patterns. The indicator methods have certain advantages over even Cartesian diver methods of determining oxygen uptake. Although they are not quantitative they are more delicate than any other method and make directly visible very slight regional differentials even in very small cells without isolation of parts. There can be no question concerning validity of the data of the present paper. With care in use of indicators and in observation, these gradient patterns can be seen by anyone not color blind and with fairly good visual perception of slight differences in depth of color. Also, with a little practice ability to distinguish slight differentials with a high degree of certainty increases.

The critic may question whether the observed gradient patterns are actually the same in different oöcytes and at different stages or whether they are merely chance differentials. In those early oöcytes showing evidence of relation to ovarian tissues the most active ends of the gradients are at or near the opposite pole. The increasingly definite relation of the nuclei to these more active gradient regions, the formation of polar bodies there and the relation of gradient patterns to physiological axes indicate their significance. In this connection it is of interest that, according to Tennent (1931), the first polar body of the sea urchin, *Mespilia globulus,* forms at the free pole of the ovarian cell.

Since this paper is concerned with gradient patterns, the occasion is taken to refer to the interesting experiments on alteration of ventrodorsality by subjection of *Dendraster* embryos at the 8-cell stage to a concentration gradient of various agents (Pease, 1941, 1942a, 1942b). Some of the agents used are known or believed to inhibit certain enzyme systems, with perhaps still other effects; actions of some others are perhaps less well known. The effective agents determined the most inhibited region as dorsal and the less inhibited became ventral with a frequency sufficient to demonstrate a positive determining action. Indicators do not show ventrodorsality in early embryonic stages, though it is doubtless present in some degree or form (see also Hörstadius and Wolsky, 1936). When it becomes visible as an indicator gradient it decreases from the ventral side dorsipetally. There is no distinguishable difference between right and left sides in early stages. Pease speaks repeatedly of his determination of bilaterality, although he actually determines ventrodorsality. Since the embryo has three spatial dimensions and since there is no evidence of asymmetry in the stages concerned, bilaterality is only an incidental consequence of determination of ventrodorsality.

In use of various agents Pease found that inhibition of cleavage and determination of ventrodorsality were not necessarily associated in action of a particular agent. To quote him concerning this point: "This leads to the conclusion that the bilateral (sic) determination is not dependent on a vague metabolic gradient but is rather dependent upon a specific enzyme system or linked systems" (Pease, 1941; p. 399). And again: "Bilateral (sic) determination by chemical concentration gradients is not dependent upon general metabolic gradients because this determination can be separated from differential cleavage inhibition" (Pease, 1942b; p. 352). The meaning of these statements is not clear. Do they mean that the ventrodorsality which he has determined is different in physiological character from the ventrodorsality of the uninhibited embryo? If this is the meaning, two different sorts of ventrodorsality can result in the same course of ventrodorsal development. This appears highly improbable. Moreover, cleavage, though to some degree correlated with gradient pattern, also unquestionably involves various activities which have little or no relation to that pattern. Some agents may inhibit these without any effect on ventrodorsality. There is every reason to believe that the experimentally determined ventrodorsality is the same as ventrodorsality in uninhibited development. Examination and counts of the material were made at the late gastrula or "prism" stage, but the question whether the experimental ventrodorsality is so completely determined that the fully developed pluteus form is attained or whether there is some differential inhibition, is not considered by Pease.

Pease also says: "The extreme sensitivity of the bilateral (!) determination to concentration gradients suggested that respiratory mechanisms might be involved (Pease, 1941). The further successful experiments with azide demonstrated with little probability of error that an oxidation system passing through a heavy metal catalyst is important in this determination" (Pease, 1942, pp. 352–3). The following pages of the summary of his paper are largely concerned with hypotheses concerning the enzyme systems involved in the determination of ventrodorsality. He seems not to be aware that according to present conceptions the action of redox indicators depends on an oxidase or oxidases and on one or more dehydrogenases. Moreover, various agents effective in determining differential developmental modifications are known to be oxidase or dehydrogenase inhibitors, or more or less general inhibitors of enzyme activity. If these respiratory enzyme systems are as important in the ventrodorsal determination as Pease maintains, there can be no doubt that the experimentally determined ventrodorsal gradient is the same physiologically as the gradient made visible by the redox indicators in uninhibited development. Incidentally, although Pease is evidently convinced that he has determined bilaterality, he does not consider how the external gradient could determine a bilaterality at a right angle to its own differential and without distinguishable difference on the two sides.

Pease's experiments suggest that a certain degree of physiological dominance of the ventral region, the high end of the ventrodorsal gradient pattern according to the redox indicators, over the less active dorsal region is present in unaltered development. With inhibition of the dominant ventral region, the dorsal region becomes more or less physiologically isolated, its activity increases and it develops as a ventral region. The experiments suggest further that in early developmental stages the difference between ventral and dorsal is at least very largely, if not entirely, quantitative.

The indicator gradients and the differential developmental modifications also suggest that in the early development of the echinoderms and of many other organisms regional differences in at least certain enzyme systems are predominantly or entirely quantitative, and that regionally specific differences gradually arise. Does not differentiation consist largely in regional localization of certain enzyme systems, while certain others remain less definitely localized? Apparently oxidases and dehydrogenases, or certain of them, are among those which usually remain rather generally distributed. In many organisms quantitative differentials in indicator patterns persist throughout life.

Most of our knowledge, as distinguished from hypotheses concerning enzymes, has been obtained by destruction of living protoplasms. We know little concerning the internal relations of enzyme systems in living, apparently uninjured organisms. The indicator methods are not quantitative but they give information which at present can be obtained in no other way. Also the differential developmental modifications give information concerning the significance of the indicator gradient patterns in developmental morphogenesis, and it appears probable that with further progress and refinement in use of external agents, they will give information concerning other than the respiratory enzyme systems.

In view of the present interest in enzyme systems it seems desirable to call attention to another aspect of action of an external agent on living organisms. In a system in which continuous correlated change is occurring, the rate of that change, in general terms the activity of the system, may be an important factor in determining its sensitivity or susceptibility to an external agent. Insofar as quantitative differences in activity are characteristic of the system or its parts, susceptibility to an external agent which retards, inhibits or otherwise alters any essential quantitative factor of the system, and so alters the system, will vary with the activity. The effect of the agent will occur more rapidly and in greater degree in the more active than in the less active system or part, irrespective of the particular character of the action of the agent. In the echinoderm eggs and embryos regional quantitative differentials in at least certain of the correlated changes characteristic of the gradient pattern are present, though not necessarily the only regional differences present. Susceptibility to any agent which acts on an essential factor of this quantitative differential will be greater in the more active than in the less active gradient levels. In other words, the action of the agent will be differential in relation to the gradient pattern; by an inhibiting agent the more active "higher" gradient levels will be more inhibited than less active levels, whatever the particular factor on which the agent acts. Also, recovery from temporary action will be more rapid and more complete at more active than at less active levels, provided action of the agent has not become irreversible, and equilibration or development of tolerance and acclimation will show the same relation to the gradient pattern. This relation of susceptibility to external agents and activity accounts for the determination of similar differential developmental modifications by many external agents which certainly do not all act in the same way on a protoplasm. And conversely, these similar modifications by different agents constitute evidence that in the echinoderms and various other organisms quantitative differentials are important factors in gradient pattern. This relation of susceptibility to activity by no means excludes the possibility that certain agents may provide evidence of specific regional differences in effect; perhaps some modifications of echinoderm development are suggestive of such effects.

The relation of susceptibility to activity holds for inorganic systems with quantitative differences in activity as well as for living organisms. For example, a rapid stream or a rapidly moving automobile is more susceptible to a sufficient degree of disturbance of any kind than a slow stream or car, and recovery from less extreme temporary disturbance and equilibration to a slight disturbance show the same relation to activity.

In conclusion, on the basis of evidence at present available it appears beyond question that a gradient pattern, primarily predominantly or entirely quantitative, is an essential factor in echinoderm larval development. Moreover, this pattern provides a physiological basis for activation of different genes in different cells or cell groups, *i.e.,* for differentiation. When this pattern is altered experimentally the course of morphogenesis is altered; when gradient pattern is obliterated, development and differentiation do not occur unless a new gradient is determined by an external agent, as has been done in certain organisms.

## SUMMARY

1. With progress in the use of redox indicators, it has been found that slight gradient differentials become more distinctly visible by intracellular oxidation of redox dyes, reduced by essentially non-toxic sodium hydrosulphite, and of low concentrations of indophenol reagents than in reduction of oxidized dyes. Staining by oxidized dyes preceding reduction may itself decrease slight differentials.

2. By means of intracellular oxidation of reduced dyes and indophenol reagents a gradient pattern has been rendered directly visible from early oöcyte to larval stages. The evidence indicates that the gradient of the early oöcyte becomes the polar gradient of the egg and embryo. In early oöcyte stages, position of the nucleus varies, but as oöcyte growth progresses it comes to lie near the pole of most rapid intracellular oxidation, the polar bodies form there and this becomes the apical (animal) pole of egg and embryo.

3. The ventrodorsal gradient pattern becomes visible in the gastrula, perhaps in the late blastula, but is undoubtedly present earlier, probably with a differential too slight to be visible or in another physiological condition. Other gradients appear in the further course of development.

4. Experiments of Pease on determination of ventrodorsality in *Dendraster* by gradients of inhibitory agents are discussed in relation to redox gradient pattern.

## LITERATURE CITED

Child, C. M., 1936a. Differential reduction of vital dyes in the early development of echinoderms. *Arch. f. Entw.*, **135**: 426–456.

Child, C. M., 1936b. A contribution to the physiology of exogastrulation in echinoderms. *Arch. f. Entw.*, **135**: 457–493.

Child, C. M., 1941. Formation and reduction of indophenol blue in development of an echinoderm. *Proc. Nat. Acad. Sci.*, **27**: 523–528.

Child, C. M., 1944. Developmental pattern in the starfish, *Patiria miniata*, as indicated by indophenol. *Physiol. Zool.*, **17**: 129–151.

Hörstadius, S., and A. Wolsky, 1936. Studien über die Determination der Bilateralsymmetrie der jungen Seeigelkeime. *Arch. f. Entw.*, **135**: 69–113.

Lindahl, P. E., 1942a. Contributions to the physiology of form generation in the development of the sea urchin. *Quart. Rev. Biol.*, **17**: 213–227.

Lindahl, P. E., 1942b. Einige Bemerkungen zu der Arbeit: "Lithium and echinoderm exogastrulation," von C. M. Child. *Protoplasma*, **36**: 558–570.

Lindahl, P. E., and H. Holter, 1940. Der Atmung animaler und vegetativer Keimhälften von *Paracentrotus lividus*. *C. R. Trav. Lab. Carlsberg, ser. chim.*, **23**: 257–287.

Loeb, J., and H. Wasteneys, 1912. Die Oxydationsvorgänge von befruchteten und unbefruchteten Seesterneier. *Arch. f. Entw.*, **35**: 555–557.

Pease, D. C., 1941. Echinoderm bilateral determination in chemical concentration gradients. I. The effects of cyanide, ferricyanide, iodoacetate, picrate, dinitrophenol, urethane, iodine, malonate, etc. *J. Exp. Zool.*, **86**: 381–404.

Pease, D. C., 1942a. Echinoderm bilateral determination in chemical concentration gradients. II. The effects of azide, pilocarpine, pyocyanine, diamine, cysteine, glutathione, and lithium. *J. Exp. Zool.*, **89**: 329–345.

Pease, D. C., 1942b. Echinoderm bilateral determination in chemical concentration gradients. III. The effects of carbon monoxide and other gases. *J. Exp. Zool.*, **89**: 347–356.

Tang, Pei Sung, 1931. The rate of oxygen consumption of Asterias eggs before and after fertilization. *Biol. Bull.*, **61**: 468–471.

Tennent, H., 1931. The maturation of the egg of the sea urchin, *Mespilia globulus*. *Science*, **74**: 574–575.

# EFFECTS OF CHEMICALS ON A SCHOOLING FISH, KUHLIA SANDVICENSIS[1, 2]

ROBERT W. HIATT, JOHN J. NAUGHTON AND DONALD C. MATTHEWS

*Dept. of Zoology and Entomology and Dept. of Chemistry, University of Hawaii, Honolulu 14, T. H.*

Recent widespread interest in the reactions of fish and other aquatic vertebrates to chemical substances has prompted us to place on record certain significant results which have accrued during the preliminary phases of an investigation directed toward dispersing schooling fish by chemical stimuli. This study was undertaken to find, by judicious selection and testing, whether or not certain chemical compounds in exceedingly dilute concentrations would evoke reactions in fish which would alter the sensory bonds between them, thus effecting the desired dissolution of the school. Although much additional research is yet to be accomplished on this problem, the results thus far point toward promising leads and will, we anticipate, be of considerable value to other workers engaged in closely related lines of investigation.

A chemo-sensory approach to the problem of dispersing fish in schools appears justified on the basis of (1) the role of vision in the formation, maintenance, and dissolution of fish schools as demonstrated by Parr (1927, 1931), Breder (1929, 1942, 1951), Bowen (1931, 1932), Breder and Nigrelli (1935), Johnson (1939), Schlaifer (1940) and others, and (2) the chemical sensitivity of fish as indicated by the early studies of Parker (1912), and a host of others up to the present day. It is clear in regard to vision that whatever may be the total forces which weld and maintain the fish school, dissolution of the school occurs when light intensity falls below a certain threshold value. Moreover, many experiments with blinded fish of species which school under normal conditions indicate that schooling fails to occur under these altered circumstances. For these reasons one of the specific objectives of this study is to discover chemical compounds capable of inducing amblyopia. As regards the chemical sensitivity of fish a search is underway to segregate from promising chemicals those which evoke the irritating or repelling responses desired. Upon this background of information further tests are being conducted with (1) closely related molecules with different substituents and (2) structurally analogous compounds. Finally, we hope to combine active parts of molecules into substances which may increase the intensity of response.

A survey of the literature indicates that the reaction of fish to chemical substances has not been a fertile field of investigation except insofar as certain specific piscicides, insecticides and pollutants are concerned. The history, use and effectiveness of piscicides have been reviewed recently by Krumholz (1948), Solman (1950) and Smith (1950). These authors stress rotenone as the most effective fish poison

---

[1] Contribution Number 29, Hawaii Marine Laboratory.

[2] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Hawaii (NR 160–165).

for use in fisheries work. Hubbs (1930) found nascent oxygen to be extremely toxic to fish, and Behrens and Hikiji (1933) discovered that central respiratory stimulants (cardiazole, camphor, picrotoxin, lobeline and rotenone) were generally toxic to fish although there is no strict structural parallelism. Throughout recorded history certain plants have been known to contain substances toxic to fish. Some of these are discussed by Fickendey (1911), Chopra (1941) and Bianco (1943). Very scant information exists on the active compounds or mode of action on fish of any of these piscicides.

Many of the newer insecticides and weed killers which are applied over extensive areas have posed problems in fish conservation. The toxicity of DDT to fish has been discussed by numerous workers, among them Ginsberg (1945, 1948) and Linduska and Surber (1948). Dilute concentrations of pyrethrum (Bandt, 1933), phenol larvicides (Knowles, Parker and Johnson, 1941), and 2,4-D (Harrison and Rees, 1946) have also been found destructive to fish.

The reaction of fish to chemicals as mediated through the olfactory sense has received considerable attention particularly since the experimental results obtained by Parker (1911, 1913), Parker and Sheldon (1914) and others who differentiated general chemical sensibility in fish into the separate receptors, stimuli and physiological effects of smell, taste and the common chemical sense. Recent studies by Hasler and Wisby (1949) have demonstrated that conditioned fish can detect phenol at concentrations of 0.0005 p.p.m. That odoriferous substances may evoke an alarm response has been demonstrated by Von Frisch (1941) who proved that the injured skin of the European minnow, *Phoxinus laevis,* gave off a substance, perceived through the olfactory epithelium, which initiated a fright response among its "school mates." Hüttel (1941) found this substance to be purine- or pterine-like. Unfortunately no quantitative data are available for comparatively assessing the response. Because of the extreme sensitivity of fish to odors, stimuli mediated through the olfactory sensations may well play an important role in the life of fish, serving to guide them to feeding grounds, to direct migrating fish to their homing areas, and to warn fish about toxic substances present in the environment (Walker and Hasler, 1949). In regard to the latter point responses of fish to pollutants have provided a considerable amount of the literature relating reactions of fish to chemical substances, especially those perceived through olfaction. The effect of phenolic substances on fish has been reviewed and extended recently by Jones (1951), toxicity levels for fish in waters polluted with hydrocyanic acid and other industrial by-products have been examined by Daugherty and Garrett (1951), and fatal concentrations of zinc for trout have been recently determined by Goodman (1951). Such responses by fish to pollutants have indicated their usefulness in the bioassay of chemical by-products (Hart, Doudoroff and Greenbank, 1945; Hasler and Wisby, 1949; Daugherty, 1951).

It is abundantly clear from the literature that the three chemical senses as described for fish by Parker (1912) are susceptible to different types of stimuli and that there is great disparity in thresholds for stimulation, especially between olfactory sensation and those of taste and the common chemical sense. Although there is no comparative information available for fish on this disparity in threshold of stimulation, tests have been conducted with humans which provide much data germane to our investigation. Katz and Talbert (1930) measured the intensities of stimulation of 55 different substances for odor, eye irritation and nasal irritation in human subjects. They found that trinitro tertiary butylxylene (artificial musk) was the most powerful as an odor and was observable at 0.00005 p.p.m., while phenacyl chloride was the most powerful eye irritant, observable at 0.0083 p.p.m.; and was also the most powerful nasal irritant, discernible at 0.021 p.p.m. It is interesting to note that the irritant receptors of the human eye are two or three times as sensitive as those in the mucous membranes in the nose, but are nevertheless 160 times less sensitive than the olfactory apparatus. At this stage in the development of a comparative physiology of the chemical senses we are not permitted to expect fish to respond to stimuli at the same threshold levels as do humans; nonetheless, it may be instructive to deduce possible proportionate threshold intensities on the part of fish from the meager data available. The maximum detectable dilution of a chemical substance in fish mediated through the olfactory receptors appears to be approximately 0.0005 p.p.m. (Hasler and Wisby, 1949). Thus, if the proportionate threshold intensities of minimal stimuli for the general chemical senses of humans and fish are somewhat comparable, we would expect that the eyes of fish might possibly be irritated by a suitable compound in a concentration as dilute as 0.08 p.p.m., and that the common chemical sensory receptors in the skin might be irritated similarly by a concentration as dilute as 0.3 or 0.4 p.p.m. These estimates provide us with a point of departure and a frame of reference for the quantitative aspects of this study.

Too little information is available to point definitely to any of the chemical senses as demanding our sole attention toward the solution of the problem. The greater sensitivity of olfactory receptors holds the possibility that certain chemicals in very dilute amounts might induce the desired response. However, none of the responses attributable to such chemicals appears to evoke behavior which would disperse schooling fish. In general, prior studies on the physiology of taste in fish have indicated that these stimuli evoke positive reactions to food, whereas negative or defensive reactions are mediated through the common chemical sense. Recent anatomical and physiological studies of the fishes *Prionotus* and *Trichogaster* indicate that the difference between the common chemical sense and taste can only be based on the innervation and the presence or absence of taste buds (Scharrer, Smith and Palay, 1947). Thus, the reaction of the animal cannot safely be used as a criterion for precise differentiation of stimulus-response patterns for these two senses. Nonetheless, the sensations resulting from stimulation of the receptors of the common chemical sense are more closely allied to the sense of pain (Moncrieff, 1944) and thus should provide the desired behavior. Since vigorous and defensive actions are the types sought in this study, the bulk of our tests involve chemicals eliciting this type of response which may be generally considered to be mediated through the free nerve endings. These, although we are not certain for this species, are presumably located over the surface of the body.

Very little information is available concerning repellent action of chemicals on bony fish. While conducting experiments on Mexican blind characins Breder and Rasquin (1943) found that acetic acid, 10 per cent, and ammonium carbonate were repellent. At the onset of World War II extensive investigations were conducted to develop a suitable shark repellent, and cupric acetate as well as other acetates was found fairly effective (Burden, 1945; McBride and Schmidt, 1943). Further analysis indicated that acetic acid was the active repelling agent when these substances were dissolved in water, but no suitable volumetric information is available. Since the War the same material was tested and found effective for sharks and several species of bony fish in Australian waters (Whitley and Payne, 1947).

## Selection of Chemicals

The basic considerations for selecting chemicals capable of dispersing schools of fish were (1) that they be grossly irritating to fish rather than merely toxic or narcotic, and (2) that they be rapid in action, reaching, in our experiments, a maximum effectiveness within a period of two or three minutes. In searching for suitable chemicals we were aware that substances which would elicit these reactions in other groups of animals might not be effective likewise for fishes. However, as a point of departure it was assumed that reactions to chemicals by other well-studied groups of animals would be helpful. Accordingly, the kinds of information which led to the initial choice of chemicals for testing were (1) actions of insecticides and insect repellents, (2) general knowledge of mammalian toxicants, stenches, irritants and lachrymators, and (3) previous results with chemical piscicides and fish repellents. After preliminary tests with substances in these categories it soon became evident that most of the desired chemical properties were obtainable in the group of irritant poisons classified usually as lachrymators and skin irritants. These substances probably impart their effect to fish through receptors of the common chemical sense as conceived by Parker. The selection of test chemicals was thus further narrowed to the classes of substances known to possess these properties from experimental results obtained for other animals, chiefly insects and mammals. Lest this system of selection miss chemicals or classes of chemicals which might be irritating to fish, but not to other animals thus far studied, other chemicals considered possibly effective have been included in the testing program.

The property of irritation is generally accorded to a chemical by the presence of characteristic groups in the molecular structure. However, an attempt to classify known irritants according to such active groups reveals many significant exceptions, so that *a priori* such a scheme does not appear very promising. Nonetheless, the many relations between structure and irritant power seem to demonstrate the anlage of a skeleton on which a classification will almost certainly be built. Other factors, such as those which control the amount of irritant reaching the sensory receptors (vapor pressure in air, and solubility in water), are certainly important contributors to the efficacy of stimulation. Upon these qualifications presumably active chemicals of the following groups have been selected for the testing program: mercaptans, sulfides, thiocyanates, isocyanates, isothiocyanates, phenols, highly halogenated compounds, and certain classes of unsaturated compounds. Those with lower molecular weight were also given priority in testing because of the greater solubility expected.

### *Classification of chemicals tested*

A preliminary classification of the chemicals tested thus far follows. It will be noted that certain of the substances listed have properties which fit them into more than one category, and thus make them doubly recommended for testing.

### a. *Insecticides and insect repellents*

Several insecticides and insect repellents have been tested. With regard to their stimulation of the common chemical sense in man these substances would fit into no single classification, but would run the gamut from bland to mildly irritating. We have explored the action of the following insecticides:

*n*-butyl carbitol thiocyanate (Lethane B 71)
chlorinated camphene (Toxaphene)
diethyl-*p*-nitrophenyl thionophosphate (Parathion)
1, 2, 3, 4, 5, 6-hexachloro-cyclohexane 92% gamma isomer or benzene hexachloride (Lindane or Gammexane)
1, 2, 3, 4, 10, 10-hexachloro-1 : 4, 5 : 8-diendomethano-1, 4, 4a, 5, 8, 8a-hexahydronaphthalene (Aldrin)
isobornyl thiocyanoacetate 80%, remainder related thiocyanoacetates (Thanite)
lauryl thiocyanate (Loro)
1, 2, 4, 5, 6, 7, 8, 8a-octachloro-4, 7-methano-3a, 4, 7, 7a-tetrahydroindane 60% with related dicyclopentadiene derivatives (Chlordane)

The following insect repellents were tested:

dimethyl phthalate
2-ethyl hexanediol-1, 3 (Rutgers 612)

From the meager structural evidence on insecticidal action, highly chlorinated organic molecules appear effective as nerve poisons, organic thiocyanates exert rapid depressant effects, and narcotic vapors such as carbon disulfide impart anaesthetic action. Thus tests were made on structurally related compounds. Halogenated compounds tested were:

alpha-chloronaphthalene
*p*-chlorophenyl isocyanate
2, 3-dichloro-1, 4-naphthoquinone
2, 4-dichlorophenol
*p*-fluorobenzonitrile
hexachlorobutadiene
phenacyl bromide
phenacyl chloride
sodium hypochlorite (Chlorox)
sodium pentachlorophenate
thiocyanic acid 5, 5-trichloro amyl ester
trifluoroacetic acid

Cyanates, thiocyanates and isothiocyanates examined were:

allyl isothiocyanate
ammonium thiocyanate
barium thiocyanate
phenyl isocyanate
phenyl isothiocyanate
potassium cyanate
potassium thiocyanate

The actions of other organic sulfur compounds noted were:

allyl thiourea
1, 2-ethane dithiol
ethyl mercaptan
hexamethylene dithiol
potassium ethyl xanthate
$\beta, \beta'$-thiodipropionitrile

The following compounds, analogous to repellents, were also tested:

dibutyl phthalate
cyclohexanol

### b. *Substances irritating or toxic to mammals*

A few compounds known to be toxic to mammals and several rated as repulsive or irritating have been tested. These were:

allyl isothiocyanate
allyl mercaptan
barium thiocyanate
caffeine
carbon disulfide
copper salts (copper chloride, sulfate and acetate)
diethylamine
dimethyl sulfate
ethyl mercaptan
isovaleric acid
methyl strychnine
Parathion
phenacyl bromide
phenacyl chloride
phenyl isocyanate
phenyl isothiocyanate
phenol
piperidine
pyridine
quinine
skatole
sodium cyanide
sodium pentachlorophenate
strychnine
thallium sulfate

### c. *Piscicides and fish repellents*

As mentioned previously, relatively scant attention has been given to the subject of reactions of fish to chemicals except for certain special purposes. The same or similar compounds found effective as piscicides or repellents by others were tested. These were:

acetic acid
benzoyl peroxide
cupric acetate
cupric chloride
cupric sulfate
ethyl mercaptan
hydrogen peroxide
phenol
rotenone (Fish-Tox)
sodium hypochlorite (Chlorox)
4-tertiary butyl catechol

### d. *Special irritants*

The following chemicals were used to test the effects of active oxygen as a possible irritant:

sodium perborate
sodium peroxide

Reactions to reduced oxygen tension were tested by adding sodium bisulfite to the water.

## Methods

### *Preparation of solutions*

Solutions of solids were prepared by weighing, and of liquids by volumetric measurement to give the required concentration (20 p.p.m., 10 p.p.m., etc.) when diluted and mixed in the known volume of sea water in the test aquaria. Solutions were made up in sea water to a volume of approximately 100 cc. Water soluble substances presented no difficulty, but for insoluble substances it was necessary to add about one drop of a dispersing agent before emulsifying with a hand homogenizer. In the early phase of the work Aerosol OT and Triton B 1956 were used as emulsifiers and dispersers for chemicals. Later a systematic search for a suitable general disperser for insoluble substances in sea water was made. Fifty commercial products submitted by the producing companies were tested for their ability to disperse mineral oil in sea water. Efficiency and general desirability were judged on the basis of the amount of foaming and the degree of precipitation or creaming after set intervals. As a result of these tests "Tergitol" Dispersant NPX was considered the most suitable under our conditions of testing. However, we are aware that a good disperser for mineral oil in sea water may not be the most satisfactory for other substances in the same medium. Final decision on emulsifiers and chemical dispersers will be made by testing them with specific compounds found to arouse the desired response in the test animals.

For preparation of solutions more dilute than 10.0 p.p.m. a definite volume of a more concentrated solution was taken, then an aliquot was introduced into the test aquaria. Compounds were screened initially at 20.0 p.p.m., and chemicals which were definitely effective at that concentration were tested at lower values. Although the common chemical sensory receptors of fish theoretically should be stimulated with appropriate chemicals at a maximum dilution of 0.3 to 0.4 p.p.m., the minimum dilution value of 20.0 p.p.m. was selected for initial screening to assure response by the fish if the substance had but slight stimulating value. Although such substances may exert no effect on fish in greater dilutions, final appraisal of the molecular structure for all active chemicals may disclose certain common characteristics which would lead us to more effective irritants and repellents, and ultimately aid in framing a structural basis of activity.

The minimum time of testing was two minutes, and observations were generally concluded at the end of that time unless the fish responded to the chemical. Although this brief interval may seem inadequate for results of such tests, it is in line with the objectives of the problem—to find a rapidly acting substance capable of dispersing schooling fish.

### *Apparatus*

The initial screening of chemical substances was conducted in aquaria of 50 liters capacity containing a school of four or five small fish, *Kuhlia sandvicensis,* each ranging from 30 to 60 millimeters in total length. The outer surface of the rear glass side of each aquarium was painted black to effect greater contrast with the silvery fish on movie film. An electrically driven stirrer was mounted in the corner of each tank to hasten the dispersal of chemical substances throughout the tank. For recording the speed of action an electric clock with an enlarged second hand was mounted above each tank. Overhead a battery of three Photoflood lamps in aluminum reflectors illuminated the contents of each aquarium.

The introduction of the chemical substance and the subsequent response on the part of the fish were permanently recorded on 16 mm. black-and-white movie film. Visual observations on behavior were also recorded. The movie record was made to detect subtle differences in reaction difficult to describe verbally or to remember for subsequent comparison.

## Results

### *Inactive chemicals*

The following list of chemicals elicited no observable response at a concentration of 20.0 p.p.m.:

acetonitrile
allyl thiourea
ammonium thiocyanate
barium thiocyanate
benzoid acid-*o*-(alpha mercaptoacetamido)
benzoyl peroxide
catechol
chlorinated camphene (Toxaphene)
alpha-chloronaphthalene
*p*-chlorophenyl isocyanate
cupric chloride
cupric sulfate
cyclohexanol
cyclohexyl-4-amino phenol HCl
gamma-chlorobutyronitrile
2-3-dichloro-1, 4-naphthoquinone
diethylamine
dimethyl phthalate
dioxane
ethylene oxide
ethylene oxide polymer
*p*-fluorobenzonitrile
hexamethylene dithiol
hydrogen peroxide
hydroquinone
$\beta$-$\beta'$-iminodipropionitrile
indole-3-acetic acid
methyl acrylate
methyl strychnine
mica (500 mesh)
morpholine
1, 2, 4, 5, 6, 7, 8, 8a-octachloro-4-7-methano-3a, 4, 7, 7a-tetra hydroindane (Chlordane)
piperidine
potassium cyanate
potassium ethyl xanthate
potassium thiocyanate
pyridine
Rutgers 612
skatole
sodium perborate
sodium peroxide
thallium sulfate
$\beta$, $\beta'$-thiodipropionitrile
triethanol amine
urea

### *Active chemicals*

Active chemicals and the degree of response by the test animals at various dilutions are summarized in Table I.

### *Effects of particle size and true solution*

Since many of the effective chemical compounds were insoluble in sea water and had to be dispersed therein in the form of an emulsion, it was necessary to know something about the relation of droplet size to the magnitude of the response evoked in the fish. Although no data are available relating the intensity of response by fish to the comparative number of sensory receptors stimulated by substances dissolved or suspended in water, we are probably permitted to assume *a priori* that the magnitude of response varies directly with the number of receptors stimulated. Thus, when fish are exposed to dilutions of relatively insoluble substances in the neighborhood of 0.1 p.p.m. it is quite possible that the number of molecules in solution or the number of particles in suspension bear significantly on the intensity of the response.

To illuminate this question for purposes of preliminary evaluation and judgment of the most potent compounds tested, the effect of particle size and true solution on the response to one of the most irritating chemicals discovered (allyl isothiocyanate) was measured. Three preparations were made. First, the dispersing agent, "Tergitol" Dispersant NPX, was added to the chemical as was sea water, followed by simple emulsification in a hand-operated homogenizer. Second, the chemical was placed in isopropyl alcohol to form a true solution to which "Tergitol" Dispersant NPX and sea water were added before emulsifying in a homogenizer. Third, a true solution of the chemical was formed by adding sea water and warming until the insoluble phase had disappeared. Droplet size of the first two emulsions was measured microscopically using an ocular micrometer. In the first suspension the droplets were quite variable in diameter, averaging 4.5 $\mu$, whereas in the second suspension they were of quite uniform size, averaging 3.0 $\mu$.

Tests were conducted as described previously using dilutions of 0.1 p.p.m. for each of the three preparations. No detectable difference in response was discerni-

TABLE I

*Summary of the responses of Kuhlia sandvicensis to various dilutions of chemical irritants. The symbol *** denotes a violent reaction, ** a medium reaction, * a slight reaction, and — no discernible reaction. See text for details.*

| Chemical | Emulsifier | Dilution in p.p.m. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 20.0 | 10.0 | 2.0 | 1.0 | 0.2 | 0.1 | 0.05 |
| Acetic acid | None | * | | | | | | |
| Acrylonitrile | None | * | | | | | | |
| Allyl isothiocyanate | None | *** | | | *** | *** | ** | — |
| Allyl mercaptan | Tergitol NPX | * | | | | | | |
| Benzene | Triton B 1956 | * | | | | | | |
| *n*-Butyl carbitol thiocyanate | None | *** | | | — | | | |
| Caffeine | Aerosol OT | * | | | | | | |
| Carbon disulfide | None | * | | | | | | |
| Catechol (derivative) | Aerosol OT | * | | | | | | |
| alpha-Chloronaphthalene | Aerosol OT | * | | | | | | |
| Cupric acetate | None | * | | | | | | |
| Dibutyl phthalate | Aerosol OT | * | | | | | | |
| 2,4-Dichlorophenol | Dioxane and Triton B 1956 | ** | | | | | | |
| Diethyl-*p*-nitrophenyl thionophosphate (Parathion) | Aerosol OT | ** | | | | | | |
| Dimethyl sulfate | None | * | | | | | | |
| 2, 4-Dinitronaphthol-ammonium salt | None | * | | | | | | |
| Du Pont PB-70 | Dioxane and Triton B 1956 | * | | | | | | |
| 1, 2-Ethane-dithiol | Triton B 1956 | ** | | | | | | |
| Ethyl mercaptan | None | *** | | — | — | | | |
| Hexachlorobutadiene | Aerosol OT and Triton B 1956 | * | | | | | | |
| 1, 2, 3, 4, 5, 6-Hexachlorocyclohexane, 92% gamma isomer (Lindane) | None | *** | | | * | | | |

TABLE I—*Continued*

| Chemical | Emulsifier | Dilution in p.p.m. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 20.0 | 10.0 | 2.0 | 1.0 | 0.2 | 0.1 | 0.05 |
| 1, 2, 3, 4, 10, 10-Hexachloro-1:4, 5:8-diendomethano-1, 4, 4a, 5, 8, 8a-hexahydronaphthalene (Aldrin) | None | *** | | — | — | | | |
| Hydroquinone | None | * | | | | | | |
| 2-Hydroxy-3-cyclo-hexyl-1, 4-naphthoquinone | Benzene and Aerosol OT | ** | | * | | | | |
| Isobornyl thiocyanoacetate (Thanite) | None | *** | *** | *** | *** | * | | |
| Isovaleric acid | None | * | | | | | | |
| Lauryl thiocyanate (Loro) | None | *** | | | | | | |
| Methyl methacrylate | None | ** | | | | | | |
| Phenol | None | *** | | ** | | | | |
| Phenacyl bromide | Ethyl alcohol and Tergitol | | | | *** | | ** | — |
| Phenacyl chloride | Ethyl alcohol and Tergitol | | | | *** | | ** | — |
| Phenyl isocyanate | Aerosol OT | ** | | | | | | |
| Phenyl isothiocyanate | None | *** | *** | | * | | | |
| Potassium thiocyanate | None | * | | | | | | |
| Quinine | Ethyl alcohol and Tergitol | | * | | | | | |
| Rotenone (Fish-Tox) | None | ** | | | — | | | |
| Sodium bisulfite | None | ** | — | | | | | |
| Sodium cyanide | None | *** | | *** | * | | | |
| Sodium hypochlorite (Chlorox) | None | | *** | *** | | — | | |
| Sodium pentachlorophenate | None | ** | | | | | | |
| Strychnine | Ethyl alcohol and Tergitol | | * | | | | | |
| 4-Tertiary butyl catechol | Aerosol OT | ** | — | | | | | |
| Thiocyanic acid 5, 5, 5-trichloro amyl ester | Tergitol | | *** | ** | | | | |
| Trifluoro acetic acid | None | * | | | | | | |

ble with the two suspensions, indicating, possibly, that differences in particle size of the magnitude mentioned do not make any appreciable difference in the reception and response to stimuli. However, the true solution did evoke a discernibly greater activity, thus possibly implying that in such dilutions molecular or near-molecular size of the irritant contributes to more widespread stimulation of available receptors.

## DISCUSSION

### *Intensity, nature and rapidity of responses*

Behavior patterns of *Kuhlia sandvicensis* in response to chemical stimulation, mediated primarily through the common chemical sensory receptors, permitted analysis by three separate criteria: (1) the intensity of response (slight, medium or violent, as indicated by the asterisks in Table I), (2) the nature of the response (the particular nervous receptors stimulated and the sensations aroused), and (3) the intervals between bodily contact with the chemical substance and the first discernible reaction. Precise quantitative analyses of the intensity of responses are impossible to achieve, but satisfactory estimates were made on a background of many tests evoking a wide variety of behaviorisms. Slight responses elicited by mild irritants were manifested by rapid mouth movements, efforts to avoid the substance, and vertical swimming, first to the surface, then down, repeated rapidly. These reactions to slight irritants occurred invariably, together with other activity, in responses rated as medium and violent, and were usually the prelude to these intensified reactions. A medium response was denoted by various patterns of behavior all more intense than that indicated for slight response, but in no case did the substances arouse violent reaction. Rapid swimming vertically and about the aquarium, gulping at the surface, jerky motion, etc., were types of responses that rated this designation. Extreme behavior of several characteristic types was designated as violent response. Very erratic and rapid swimming, often leaping out of the aquarium, paralysis, head shaking, blindness and death within two or three minutes were classified in this category.

The mode and place of action of chemicals on fish are not simple to define. The widespread distribution of common chemical sensory receptors on the general body surface, eyes, nasal capsules and the mouth, coupled with the fact that the complexes of chemical senses are not well differentiated for fish, behavioristically speaking, as they are for mammals, precludes the expectation of precise patterns of response to particular stimuli. Excluding the chemical senses of taste and smell, we would still expect different modalities of the common chemical sense in fish just as are found in humans, where different sensations are experienced from substances attacking the eyes, the nose or the throat. An analysis of the responses by fish to chemicals known to arouse particular sensations in other animals bears out this supposition. For example, well known general skin irritants for man such as phenol and allyl isothiocyanate apparently stimulate all free nerve endings on the body making it impossible to pinpoint a site of irritation because of the violent reaction evoked. Also, the very potent lachrymators, phenacyl bromide and phenacyl chloride, arouse intense sensations resulting in violent head shaking and early indications of blindness, precisely the mode of response expected for substances irritating primarily to the eyes. Sodium cyanide and thiocyanic acid 5, 5, 5-trichloro amyl ester, substances related to respiratory poisons for insects (Brown, 1951), and the reducing agent, sodium bisulfite, caused fish to stay at the surface where they gulped air and showed other typical signs of suffocation. Both tetanic and flaccid paralytic effects were noted for certain compounds tested. A clear example of tetanic paralysis wherein the operculum was fixed rigidly at an acute angle on the head, the fins held stiffly outward, and the body bent in a convulsive manner resulted from the introduction of isobornyl thiocyanoacetate. This substance is known to act as a narcotic for insects but induces paralysis in mammals. Ethyl mercaptan, of which little is known neurophysiologically except for its effect on the olfactory receptors, also induced a severe tetanic paralysis in fish. Notable among those substances inducing flaccid paralysis was thiocyanic acid 5, 5, 5-trichloro amyl ester, which, in addition, destroys the equilibrium. The slowly moving fish gradually leave the surface, where they also exhibit symptoms of suffocation, and swim upside down before they eventually become moribund on the bottom of the tank.

Characteristic responses which appeared to differentiate between stimulation of taste receptors and of the common chemical sense were aroused by testing quinine and strychnine. These substances, according to Scholl and Munch (1937), are two of the most bitter substances stimulating the human taste, strychnine being about three times as bitter as quinine. Brucine, about three to four times as bitter as strychnine, was not available for testing. Test fish shook their heads violently and made "spitting" motions for a few seconds after the initial contact with the alkaloids, then, exhibiting no irritation, settled down to normal schooling behavior. Quinine actually evoked a more intense and more lengthy response than strychnine.

### *Comparative responses of fish and other animals to chemical irritants*

Good examples of the highly selective nature of the sensations aroused by chemicals on different organisms are clearly shown in the list of inactive chemicals and in the table of active compounds. Although there was some relationship between chemicals irritating to fish and those having a similar effect on other animals, there were many and significant exceptions.

Exceptions in the ranking position of the chemicals tested thus far were the high molecular weight thiocyanates which had little or no effect on the olfactory and common chemical sensory receptors of mammals, and may even have a relatively low order of toxicity, but are classed here as some of the most effective irritants to fish. A possible explanation for this disparity in reactivity may be associated with the medium in which the animals exist, air in the case of mammals and water in the case of fish. In the former, where air transport is necessary, the relatively low vapor pressure would seem to be a plausible explanation of the lack of response. In the latter, greater reactivity for fish probably results from relatively greater solubility, an assumption which awaits confirmation. Substances of this character emphasize the necessity of an empirical search through a wide variety of chemical types for compounds which, although not irritating to other animals, may arouse intense sensations in fish.

An appreciable number of the chemical groups tested were selected because they elicit a profound and often violent response in man, but we have found them rather ineffective as fish irritants. Such chemicals as most mercaptans, certain amines, some nitriles and skatole fell into this category. For an explanation of much of this disparity in reaction we concur with Moncrieff (1944) that in man conditioned reflexes are important, resulting from associating many of these odors with unpleasant experiences or with substances which we learn to recognize as toxic. Significant also, we suspect, is the fact that most of these substances have a low solubility in sea water, thus having a sub-minimal concentration for reaction, whereas nearly all have an appreciable vapor pressure.

Substances which were found to have about equal effect in irritating fish and mammals fall generally into the groups of intense skin irritants and lachrymators, examples of which are allyl isothiocyanate, phenacyl chloride and phenacyl bromide. Noteworthy among these generally effective compounds is the prevalence of sulfur, which lends support to the point stressed by Moncrieff (1944) that this element imparts skin-penetrative power, thus increasing the vulnerability of the free nerve endings. Our results also emphasize the importance of the bond type, whether it be ionic or covalent, in governing the effect of irritants. For example, all thiocyanates tested were effective irritants in the organic covalent form, but the ionic salts of ammonium, barium and potassium thiocyanate were ineffective. Conversely, covalent cyanides or nitriles proved ineffective, but the cyanide ion belonged with the group of irritants.

Substances found most effective in repelling sharks and certain bony fishes (Burden, 1945; Whitley and Payne, 1947), cupric acetate and acetic acid, were only mildly irritating to our test species and show little promise of satisfying the objectives of this study.

It was pointed out previously that too few data were available in the literature to formulate conclusions on comparative stimulus-response activity in the chemical senses between humans and fish, but that the ratio of response to strength of stimulus for the complex of chemical senses in humans was rather well known, especially since the researches of Katz and Talbert (1930). A hypothetical working parallelism for the chemical senses in fish was established on the basis of the maximum detectable dilution of a chemical substance in fish mediated through the olfactory receptors, based on the results of Hasler and Wisby (1949). We calculated, in accord with the proportional stimulus-response data for the chemical senses in man, that the eyes of fish might possibly be irritated with suitable chemicals at a dilution of about 0.08 p.p.m., and that the common chemical sensory receptors over the remainder of the body might be stimulated at a maximum dilution of about 0.3 or 0.4 p.p.m.

Our data indicate that both phenacyl bromide and phenacyl chloride, known to be powerful lachrymators and to induce behavior in our experimental animals suggestive of intense irritation of the eyes, were quite effective in a dilution of 0.1 p.p.m. and ceased to be discernibly effective at 0.05 p.p.m., precisely fitting our hypothesis. The most effective general irritant, allyl isothiocyanate, evoked observable non-localized responses at the level indicated for the two lachrymators just discussed. The next most effective general skin irritant, isobornyl thiocyanoacetate, failed to arouse discernible sensations at dilutions below 0.2 p.p.m.

### *Solubility of organic compounds in sea water*

Our results have pointed to the fact that the distribution of the reacting substances in sea water, either in the form of a true solution or a colloidal sol, is basic to stimulus-response investigations of this type. These data, although only suggestive in nature, seemed to indicate that finely dispersed particles, particularly in the less concentrated solutions, were not as effective for the stimulation of the free nerve endings over the general body surface as molecules in a true solution. Clouding this aspect of the work is the near absence of solubility data for organic compounds in sea water. However, we can extend what is known about the solubility of these compounds in pure water in an effort to predict their behavior in sea water. It is to be expected that solubility will be generally less in sea water because of the "salting out" effect. Moreover, we would expect in any group of organic compounds having closely related structural composition that the solubility would be greater for those of lower molecular weight and of greater polarity. Considerations such as these guided our selection of compounds.

Observations on the use of chemical dispersing agents with rather insoluble compounds indicated, because of the persistence of a separate phase of emulsion droplets in very dilute solutions where the concentration was well below the amount capable of existing in true solution, that the film of emulsifying agent surrounding each droplet may inhibit the attainment of equilibrium between the two phases of solvent and solute. Because of this, caution must be used in selecting and using chemical dispersing agents when it is desired to achieve maximum molecular concentration of the solute.

### *General conclusions of a preliminary nature*

Although generalizations at this stage of development in this project may be somewhat premature, certain aspects appear reasonably clear. An evaluation of the behavior of the test schools in response to the active chemicals definitely indicated that substances perceived only through the human chemical receptors as odors or tastes did not arouse sensations capable of dispersing schooling fish, but that substances which stimulate human receptors for the common chemical sense, such as general skin irritants, lachrymators and nerve poisons, were effective. Thus future work will be directed toward securing more potent irritants and toward developing synergistic reactions with those already proven effective. A listing of the types of compounds expected to be most effective, based on our analysis of eighty-seven chemicals tested thus far, and placed in order of probable importance, includes multi-halogenated organic compounds, organic thiocyanates, organic isothiocyanates and halogenated ketones. Revisions of this list may be expected as further tests are made within each group, as our list of groups tested is extended, and as other species of schooling fish are used as test animals. We wish to point out again that, although the results of this investigation have been secured by testing and analyzing the reaction of fish to 87 judiciously selected compounds, these appraisals are considered preliminary because of the vast amount of work yet to be done along the lines suggested by this phase of the investigation.

The authors are indebted to many chemical companies which have offered chemical dispersing agents and chemical samples for testing and greatly appreciated advice.

## SUMMARY

1. The objective of this study was to find, by judicious selection and testing, whether or not certain chemical compounds in exceedingly dilute concentrations would arouse sensations in fish which would alter the sensory bonds between them, thus effecting dispersal of schooling fish.

2. The basic considerations in selecting chemicals capable of dispersing schools of fish were (1) that they be grossly irritating to fish rather than merely toxic or narcotic, and (2) that they be rapid in action, reaching, in our experiments, a maximum effectiveness within a period of two or three minutes.

3. A preliminary classification of the 87 compounds tested included insecticides and insect repellents, substances irritating or toxic to mammals, piscicides and fish repellents, and special irritants. Suitable chemical dispersers were added to the relatively insoluble compounds. Tests were made at dilutions of 20.0, 10.0, 2.0, 1.0, 0.2, 0.1, 0.05 p.p.m.

4. The intensity, nature and rapidity of response to introduced chemicals were recorded by observation and by motion pictures. Although a clear perceptual pattern of the mode and site of action of certain chemicals known to arouse localized sensations in terrestrial animals was not definitely discernible, certain reactions suggested that special areas were intensely stimulated. General skin irritants (phenol, allyl isothiocyanate) strongly stimulated the entire body; lachrymators (phenacyl chloride, phenacyl bromide) seriously irritated the eyes and impaired vision; some general nerve poisons (isobornyl thiocyanoacetate, ethyl mercaptan) induced tetanic paralysis; whereas another (thiocyanic acid 5, 5, 5-trichloro amyl ester) resulted in flaccid paralysis; and respiratory impairment was brought about by a reducing agent (sodium bisulfite) and by probable respiratory poisons (sodium cyanide, thiocyanic acid 5, 5, 5-trichloro amyl ester).

5. Examples of the highly selective nature of the sensations aroused by chemicals on different organisms were clearly demonstrated. High molecular weight thiocyanates which have little or no effect on the common chemical sense of mammals are highly effective irritants for fish. Others (mercaptans, certain amines, some nitriles, skatole), exceedingly irritating to man, appear to have little or no effect on fish. Substances classed as intense skin irritants or as lachrymators (allyl isothiocyanate, phenacyl chloride, phenacyl bromide) for man appear to be equally effective for fish. Underlying reasons for such disparities and similarities in reaction were suggested.

6. Certain characteristics of molecules appear to be significant in predicting success for irritants, and thus contribute toward a framework for a theory establishing relations between chemical constitution and physical properties on the one hand and aggressive action on the other. Increased aggressiveness with sulfur in the molecule was noted, and the bond type, ionic or covalent, apparently exerts a governing effect with irritants as shown by contrast between the very effective covalent thiocyanates and the ineffective ionic thiocyanate salts.

7. A striking proportionate parallelism seems to exist for stimulus-response reactions of all the chemical senses between humans and the fish used for these tests.

8. Results thus far indicate that substances perceived as odors and tastes for humans do not arouse sensations capable of dispersing fish in schools, but that general skin irritants, lachrymators and nerve poisons may be capable of doing so. Types of compounds which now seem most effective for dispersing fish in schools are multi-halogenated organic compounds, organic thiocyanates, organic isothiocyanates and halogenated ketones.

## LITERATURE CITED

Bandt, H. J., 1933. Fish poisoning caused by pyrethrum. *Mitt. Fischereiver. Wasser u. Abwasser,* **31**: 255.

Behrens, B., and K. Hikiji, 1933. Comparative studies on the toxicity of respiratory stimulants for fish. *Arch. intern. Pharmacodynamie,* **46**: 233–238.

Bianco, J. G., 1943. Plants that are poisonous to fish: Venezuelan species. Pharmacology of rotenone. *Gaceta farm.,* **3**: 22–40.

Bowen, Edith S., 1931. The role of the sense organs in aggregations of *Ameiurus melas. Ecol. Monogr.,* **1**: 1–35.

Bowen, Edith S., 1932. Further studies on the aggregating behavior of *Ameiurus melas. Biol. Bull.,* **63**: 258–270.

BREDER, C. M., JR., 1929. Certain effects in the habits of schooling fishes, as based on the observations of *Jenkinsia*. *Amer. Mus. Novitates, No. 382: 1–5.*

BREDER, C. M., JR., 1942. Social and respiratory behavior of large tarpon. *Zoologica,* **27**: 1–4.

BREDER, C. M., JR., 1951. Studies on the structure of the fish school. *Bull. Amer. Mus. Nat. Hist.,* **98**: 1–27.

BREDER, C. M., JR., AND R. F. NIGRELLI, 1935. The influence of temperature and other factors on winter aggregations of the sunfish, *Lepomis auritus,* with critical remarks on the social behavior of fishes. *Ecology,* **16**: 33–47.

BREDER, C. M., JR., AND PRISCILLA RASQUIN, 1943. Chemical sensory reactions in the Mexican blind characins. *Zoologica,* **28**: 169–200.

BROWN, A. W. A., 1951. Insect control by chemicals. John Wiley & Sons, New York.

BURDEN, W. D., 1945. Development of a shark deterrent. *Air Surgeon's Bull.,* **2**: 344–347.

CHOPRA, R. L., 1941. Insecticidal and piscicidal plants of India. *J. Bombay Nat. Hist. Soc.,* **42**: 854–902.

DAUGHERTY, F. M., JR., 1951. A proposed toxicity test for industrial wastes to be discharged to marine waters. *Sewage and Industrial Wastes,* **23**: 1029–1031.

DAUGHERTY, F. M., JR., AND J. T. GARRETT, 1951. Toxicity levels of hydrocyanic acid and some industrial by-products. *Texas Jour. Sci.,* **3**: 391–396.

FICKENDEY, E., 1911. Kamerus fish poisons. *Zeitschr. angew. Chem.,* **23**: 2166–2167.

FRISCH, K. VON, 1941. Ueber einen Schreckstoff der Fischhaut und seine biologische Bedeutung. *Zeitschr. f. vergl. Physiol.,* **29**: 46–145.

GINSBURG, J. M., 1945. Toxicity of DDT to fish. *J. Econ. Entomol.,* **38**: 274–275.

GINSBURG, J. M., 1948. An evaluation of the new toxicants for mosquito control in New Jersey. *Proc. New Jersey Mosquito Extermination Assoc., 35th Ann. Meeting, 28–35.*

GOODMAN, J. R., 1951. Toxicity of zinc for Rainbow Trout (*Salmo gairdnerii*). *Calif. Fish and Game,* **37**: 191–194.

HARRISON, J. W. E., AND E. W. REES, 1946. 2, 4-D toxicity. 1. Toxicity towards certain species of fish. *Amer. Jour. Pharm.,* **118**: 422–425.

HART, W. B., P. DOUDOROFF AND J. GREENBANK, 1945. The evaluation of the toxicity of industrial wastes, chemicals, and other substances to freshwater fishes. Contr. Waste Control Lab., Atlantic Refining Co., Phila.

HASLER, A. D., AND W. J. WISBY, 1949. Use of fish for the olfactory assay of pollutants (phenols) in water. *Trans. Amer. Fish. Soc.,* **79**: 64–70.

HUBBS, C. L., 1930. The high toxicity of nascent oxygen. *Physiol. Zool.,* **3**: 441–460.

HÜTTEL, R., 1941. Die chemische Untersuchung des Schreckstoffes aus Elritzenhaut. *Naturwiss.,* **29**: 333–334.

JOHNSON, W. H., 1939. Effects of light on movements of herring. *J. Fish. Res. Bd. Canada,* **4**: 349–354.

JONES, J. R. E., 1951. The reactions of the minnow, *Phoxinus phoxinus* (L.), to solutions of phenol, ortho-cresol and para-cresol. *J. Exper. Biol.,* **28**: 261–270.

KATZ, S. H., AND E. J. TALBERT, 1930. Intensities of odors and irritating effects of warning agents for inflammable and poisonous gases. *U. S. Dept. Comm. Bur. Mines Tech. Pap., No. 480.*

KNOWLES, F. L., W. V. PARKER AND H. A. JOHNSON, 1941. "Phenol" larvicides for mosquito control. *U. S. Pub. Health Repts.,* **56**: 1637–1641.

KRUMHOLZ, L. A., 1948. The use of rotenone in fisheries research. *Wildl. Mgt.,* **12**: 305–317.

LINDUSKA, J. P., AND E. W. SURBER, 1948. Effects of DDT and other insecticides on fish and wildlife. Summary of investigations during 1947. *U. S. Dept. Int. Fish and Wildl. Ser., Circ. No. 15.*

MCBRIDE, A. F., AND A. H. SCHMIDT, 1943. Final report on shark repellent experiments conducted in Ecuador. *Comm. on Medical Res., Off. Sci. Res. Dev.* (declassified).

MONCRIEFF, R. W., 1944. The chemical senses. John Wiley & Sons, New York.

PARKER, G. H., 1911. The olfactory reactions of the common killifish, *Fundulus heroclitus* (Linn.). *J. Exp. Zool.,* **10**: 1–5.

PARKER, G. H., 1912. The relation of smell, taste and the common chemical sense in vertebrates. *J. Acad. Nat. Sci., Phila., Ser. 2 (15):* 219–234.

PARKER, G. H., 1913. The directive influence of the sense of smell in the dogfish. *Bull. U. S. Bur. Fish.,* **33**: 61–68.

PARKER, G. H., AND R. E. SHELDON, 1914. The sense of smell in fishes. *Bull. U. S. Bur. Fish.*, **32**: 33–46.

PARR, A. E., 1927. A contribution to the theoretical analysis of the schooling behavior of fishes. *Occ. Pap. Bingham Oceanogr. Coll.*, **1**: 1–32.

PARR, A. E., 1931. Sex dimorphism and schooling behavior among fishes. *Amer. Nat.*, **65**: 173–180.

SCHARRER, E., S. W. SMITH AND S. L. PALAY, 1947. Chemical sense and taste in the fishes, *Prionotus* and *Trichogaster*. *J. Comp. Neurol.*, **86**: 183–198.

SCHLAIFER, A., 1940. The locomotor activity of the goldfish, *Carassius auratus* L., under various conditions of homotypic and heterotypic grouping. *Ecology*, **21**: 488–500.

SCHOLL, F. M., AND J. C. MUNCH, 1937. Taste tests. IV. Relative bitterness. *J. Amer. Pharm. Assoc.*, **26**: 127–129.

SMITH, M. W., 1950. The use of poisons to control undesirable fish in Canadian freshwaters. *Canadian Fish Culturist, No. 8.*

SOLMAN, V. E. F., 1950. History and use of fish poisons in the United States. *Canadian Fish Culturist, No. 8.*

WALKER, T. J., AND A. D. HASLER, 1949. Detection and discrimination of odors of aquatic plants by the bluntnose minnow (*Hyborhynchus notatus*). *Physiol. Zool.*, **22**: 45–63.

WHITLEY, G. P., AND G. H. PAYNE, 1947. Testing a shark repellent. *Australian Zool.*, **11**: 151–157.

# STUDIES ON THE ELASMOBRANCH KIDNEY. II. REABSORPTION OF UREA BY THE SMOOTH DOGFISH, MUSTELUS CANIS

RUDOLF T. KEMPTON

*Department of Zoology, Vassar College, Poughkeepsie, N. Y., and Marine Biological Laboratory, Woods Hole, Mass.*

There has been evidence of reabsorption of urea by the elasmobranch tubules for over forty years. The fact that the blood and tissues contain an unusually high concentration of urea has been known since 1859, and the discovery that the urine contains a much lower level of this material was made as early as 1906. There is, therefore, a wide variation in the way in which the various kidneys treat urea: in the mammal, a back diffusion, perhaps with other factors involved (Shannon, 1936); in the aglomerular fish, probably an inward diffusion (Marshall, 1930); in the frog, secretion (Marshall, 1932; Walker and Hudson, 1937); and in the elasmobranchs, active reabsorption.

There appears to have been no adequate inquiry concerning the detailed behavior of the elasmobranch kidney in relation to urea, despite the long existence of evidence of its reabsorption and its quantitative demonstration by Clark and Smith (1932).

There has been no demonstration of the site of urea reabsorption. It was suggested by Marshall (1930) and by Smith (1931) that the "special segment" of the elasmobranch tubule is responsible for urea reabsorption. This has been accepted by Baldwin (1937) and other writers. However it has been shown by direct puncture methods that this special segment does not exist in the tubule of the spiny dogfish, *Squalus acanthias* (Kempton, 1943). Moreover there is reason to believe that the concept of a special segment is a mistake and that the segment does not exist in any elasmobranch tubule.

Attention now has been turned to a study of urea reabsorption by the entire kidney with the hope that such an investigation might give some clue concerning the reabsorptive mechanism. This primary objective was successful to the extent that some of the factors controlling the rate of urea reabsorption have been revealed. Conclusive evidence as to the site of reabsorption in relation to other tubule functions has not been found.

## MATERIALS AND METHODS

The simultaneous reabsorption of water, urea and glucose has been studied, using inulin as a measure of glomerular filtration. Clearances were calculated by use of the well-known method of Smith (1939): UV/P, in which U is the mgm.% of the material in the urine, V is the volume of urine in cc./hr./k., and P is the mgm.% present in the plasma. Contrary to the usual practice in urea studies, plasma concentrations instead of whole blood were used because of the finding by Dr. Arthur K. Parpart (personal communication) that the erythrocytes of this species are only slowly permeable to urea.

For inulin determinations the resorcinol method of Hubbard and Loomis (1942) was employed, with two modifications. Since it was more convenient to use larger cuvettes in the spectrophotometer, 2.0 cc. samples of the plasma or urine filtrate diluted to the range of the method were used, with the final dilution in sugar tubes to the 12.5 cc. level. Heating was extended from 8 to 16 minutes, a change which produced somewhat more constant results. The tubes were read against a blank of the reagents in distilled water. Use of other blanks recommended by Hubbard and Loomis was tried repeatedly, but since no effect was ever found their use was dropped in the routine analyses. The method as modified was found to be very satisfactory, and it was not affected even by elevated glucose levels. Special tests indicated that interference from this source did not appear until the glucose level in the diluted plasma or urine filtrate was raised to about ten times the concentration of inulin.

The urea method was a slight modification of the urease and direct Nesslerization method of Koch and Hanke (1948). Because this method was developed for the relatively low plasma level and the high urine level of mammals, several changes had to be introduced to care for the reversed situation found in the elasmobranchs. Plasma was first diluted to range (usually a 20 × dilution) and then digested with Koch's glycerol urease extract at 50° C. for 60 minutes. In preparing the filtrate, the sodium tungstate and sulfuric acid were reduced in amount to 0.5 cc. each, and the normality of the acid was raised to 1.0. After filtration and Nesslerization, the readings were made against ammonium sulfate standards. Urine was treated by the same method, except that the initial dilution was usually 6 ×. The method as used includes any ammonia nitrogen which might be present. It was deemed inadvisable to attempt to separate the two sources of nitrogen because there might be some urea decomposition during the prolonged collection periods, and in addition Denis (1922) found very little ammonia nitrogen in elasmobranchs.

The method as modified was satisfactory for the purpose, but it had two objectionable features: the high dilution magnified very greatly any differences in the spectrophotometric readings; for some reason a blank containing urease gave an undue amount of light scatter. In a few cases the urine samples also gave excess scatter and had to be discarded. The source of this effect was not determined.

For glucose analyses the arsenomolybdate method of Nelson (1944) was selected. Undiluted plasma filtrates and diluted (4 ×) unfiltered urine were used. The blank consisted of the normal reagents in distilled water.

All readings were made with a Coleman Junior Spectrophotometer with cuvettes 19 mm. in diameter. Wave-lengths used were as follows: inulin method, 495 millimicra; urea, 560; glucose, 500.

Only female dogfish were used. This was due partly to the small number of males actually caught, and partly to possible complications resulting from the use of the same ducts for seminal fluid and urine. The weight of the experimental fish ranged from 5.3 to 10.3 kilograms. Below this range the urinary papilla was so small that urine collections were impracticable; at the top weight the animals were so strong that it was barely possible to handle them without damage to the balloon which collected the urine. During May and June animals of the necessary size were obtained from the traps of commercial fishermen, but after about the first of July it was necessary to catch them by hook after sunset either in deep water by the shore or from a boat in the deeper water of the harbor. The fish were always transferred immediately to a tank of fresh sea water and rushed to a live car in a float, by which tidal currents swept at a rate sufficient to ensure rapid and complete change of water (or, in 1950, to a large concrete tank with well aerated running water). The animals refused even living food in captivity; in fact they were very likely to regurgitate food which had been taken before capture. In a few cases the hook wound continued to bleed, especially when the animals were handled for collecting blood and urine. (These animals were discarded.) At best the life in captivity was limited to a few days, and there was some evidence that this was in part a temperature effect. As the water temperature became higher during the summer their life expectancy became reduced. The warmer waters of the Eel Pond in mid-summer killed dogfish in a few hours. A period of exceptionally hot weather had a very deleterious effect on the animals in use at the time. The more constant temperature in the concrete tank allowed the animals to remain in good condition for several days longer than when the live car was used. Usually the experiment was ended when the urinary papilla became so damaged that it would no longer support a catheter. The animals usually lived several days after the enforced termination of the collections. It was only in the most prolonged experiments that there was any appreciable reduction in the blood sugar level.

The animals were prepared for the collection of urine by the removal of the tip of the urinary papilla, and the insertion into the papilla of an in-lying catheter of glass. A rubber balloon, tied securely to glass tubing, was anchored by ligature to the ventral surface of the tail. The catheter was connected with the glass tubing by a piece of flexible rubber tubing, with sufficient slack to permit swimming and movements of the cloacal region without placing a strain on the catheter and the papilla. At the end of each collection period, the ligature holding the balloon was cut, the tubing disconnected from the catheter, and the urine poured into a collecting vessel. The abdomen was massaged carefully to force from the kidney ducts any fluid they might contain, this urine being removed from the catheter by syringe. The amount obtained by massage usually varied from zero to five cubic centimeters, although in one very exceptional case 40 cc. was collected. The balloon was then replaced by a fresh one. These balloons were of sufficient size to ensure that no back-pressure developed. These and other manipulations were performed on the float containing the live car, or beside the concrete tank. This made it possible to transfer the animal quickly from the circulating water to a trough, where it was tied on its back with its head immersed in fresh sea water. No anesthetic was ever used.

Administration of various materials was by diverse routes. Inulin, prepared as a 20% solution in warm distilled water, was injected intravenously through the caudal blood vessel. In some experiments other materials were also injected in the same way. Water was sometimes given by stomach tube, but this was not the standard procedure. Urea was administered in three different ways. Intravenous injections were fatal quickly. In a number of cases intraperitoneal injections were also fatal, but more slowly, with the liver exhibiting severe lesions. Intramuscular injections were partially successful, since an increase of plasma urea levels ranging up to 50% was obtained in about half of the injected animals. The firm unyielding nature of the flesh made difficult the injection of more than a few cubic centimeters at any one point. It was necessary therefore to make a series of small injections along the epaxial muscles of both sides. The dosage was 10 cc. per kilogram of a

50% solution in distilled water, freshly prepared at room temperature. In some cases this dosage was repeated twice, giving a total urea administration of 15 grams of urea per kilogram of body weight.

Blood was collected by syringe in amounts of 10 cc., and was transferred at once to test tubes containing 0.1 mgm. potassium oxalate, precipitated by drying from a 20% solution. Upon return to the laboratory from the float or tank this oxalated blood was centrifuged immediately, the plasma either being used at once or being stored over night in a very cold refrigerator. When haematocrit readings were made, capillary tubing of uniform internal diameter of 0.6 mm. was used, the centrifugation being for one-half hour in an International clinical centrifuge using

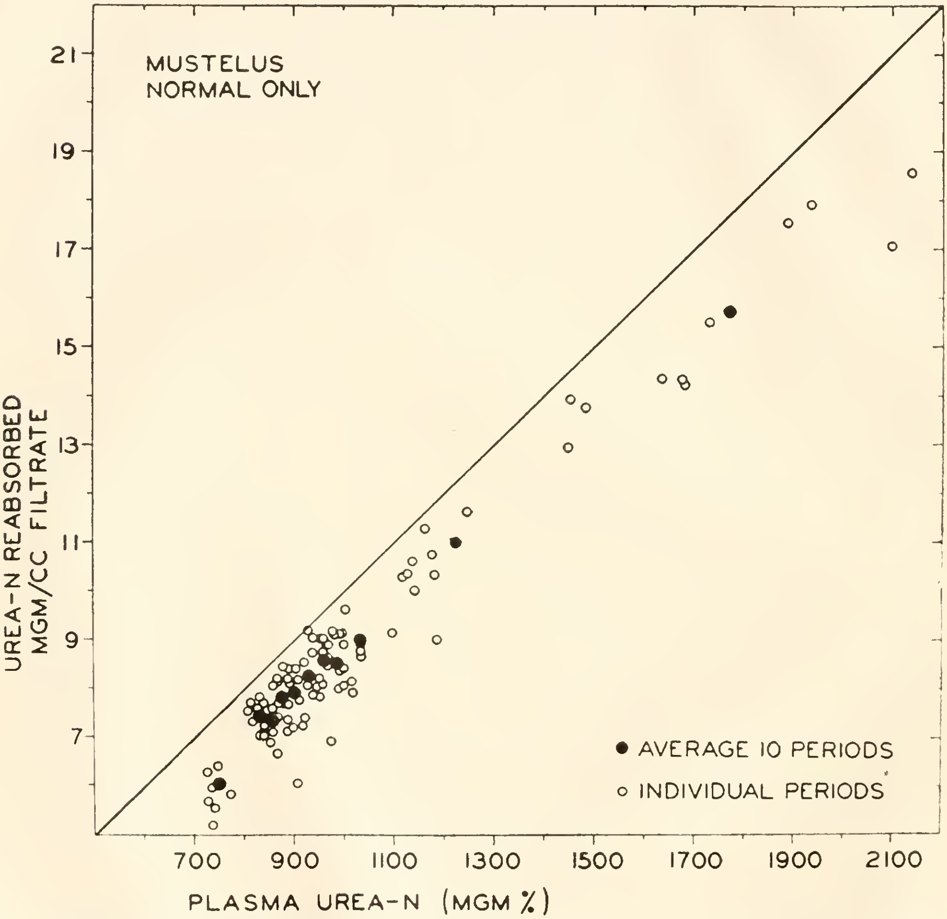


FIGURE 1. Relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea level; 110 collection periods from smooth dogfish in good condition. The abscissa could be labelled equally well "Filtrate urea-N (mgm.%)" instead of referring to plasma level. For each point, the vertical distance to the diagonal line represents the unreabsorbed moiety; the vertical distance extrapolated to a zero baseline represents the reabsorbed urea.

direct current. Top speed of 3000 r.p.m. gave a centrifugal force of 1600 × gravity. This force, for this period of time, gives as complete packing as an air turbine developing 20,000 × gravity (according to turbine determinations by Dr. James W. Green).

The routine plan of the experiments, from which there was considerable deviation, called for the capture of the fish one evening, its weighing the next morning, the injection of inulin early that afternoon, and the start of the first collecting period the next morning. Collections of blood were made at the beginning and end of each period. Urine was collected at the end of the period. Usually collections were made morning, early afternoon and evening, giving periods of approximately 12, 6, and 6 hours. In many cases a midnight collection was made also, thus dividing the day approximately into four six-hour periods. The delay between the injection of the inulin and the start of the first collection period placed all periods on a fairly flat portion of the inulin tolerance curve.

## RESULTS

There is a linear relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea concentration (or the filtrate concentration). In general, regardless of the concentration of urea in the plasma and filtrate, all of the urea is reabsorbed except a constant residuum which stays in the tubule. This relationship is true on the average (Fig. 1), the minor variations in the unabsorbed urea being related to other factors as indicated below. This general relationship holds true over a range of normally occurring plasma levels in which the highest is approximately triple the lowest. Of all the data obtained, this average level of the unabsorbed urea in the tubules is the only constant factor. Raising the urea level, in an attempt to determine whether there is a definite tubular maximum at which the relationship would no longer hold, met with failure. Fortunately the range of normal variation of plasma level was sufficiently great to permit the linear relation to appear.

From *a priori* considerations there are a number of other correlations which might be expected. Surprisingly these do not appear and some of these negative results seem to have interpretative significance. Factors which show no correlation include the following combinations:

(1) Plasma urea level (mgm.%) and urea reabsorption (mgm./hr./k.)
(2) Plasma urea level (mgm.%) and urea filtered (mgm./hr./k.)
(3) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./hr./k.)
(4) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./cc. filtrate)
(5) Urea reabsorption (mgm./hr./k.) and glucose reabsorption (mgm./hr./k.)
(6) Inulin U/P ratio and urea U/P ratio
(7) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./hr./k.)
(8) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(9) Plasma urea level (mgm.%) and % urea reabsorbed
(10) Glucose reabsorbed (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(11) Urine urea level (mgm.%) and urea reabsorbed (mgm./cc. filtrate)

There is no reason to believe that the lack of correlation is due to incomplete emptying of the kidney ducts, although this is theoretically possible in some cases.

In the first place, every effort was made to massage all the urine from the ducts. Secondly, when the data are in terms of a unit volume of filtrate, as in 4, 8, 10 and 11 above, uncollected urine could have only a very slight effect on the calculations.

The scatter in the data is not merely fortuitous. While the main amount (average 87%) of urea reabsorption from each cubic centimeter of filtrate is correlated with the plasma (and filtrate) level, the variations in unabsorbed urea are correlated with water reabsorption (Fig. 2), urine flow (Fig. 3), and urine urea concentration (Fig. 4). In other words, the variations of unabsorbed urea are correlated with the behavior of the tubules toward water, at least in part, with more urea remaining in the tubules unabsorbed when there is less water reabsorption, a higher urine rate, and concomitantly a higher concentration of urea in the urine. It should be pointed out that these variations in unabsorbed urea are definitely not correlated with differences in the rate of filtration, of glucose reabsorption, or the absolute rate of water or urea reabsorption. This will be referred to in the discussion.

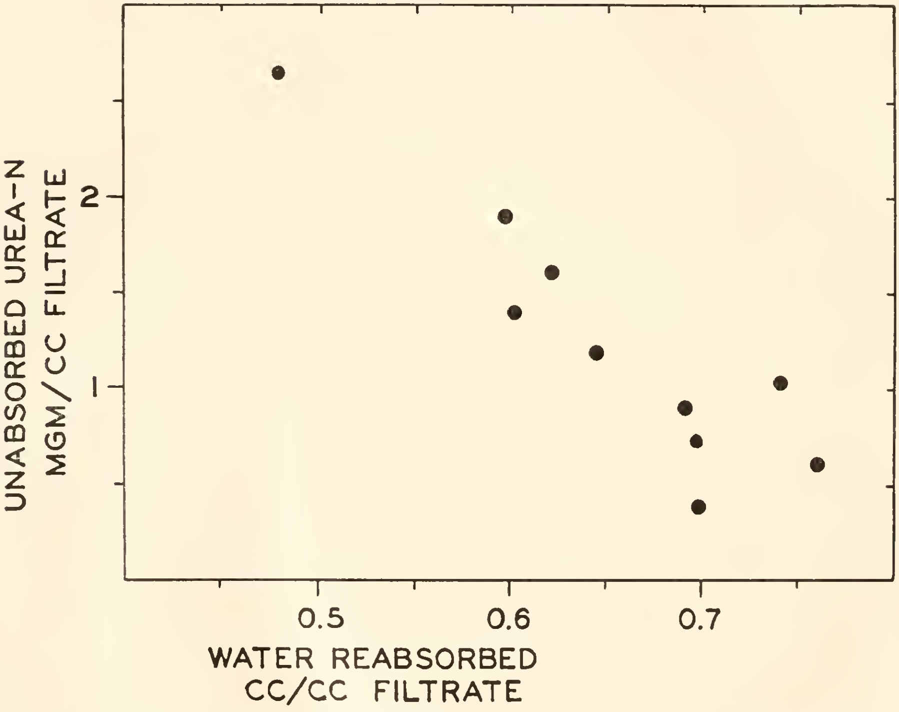


FIGURE 2. Variation in the unabsorbed urea in relation to the amount of water reabsorbed, both in terms of each cubic centimeter of filtrate. Each point represents the average of 10 collection periods.

As mentioned previously, an attempt was made to raise the urea level of the blood above the normal range. This proved to be difficult. One great obstacle was the fact that following urea injections the animals tended to continue to bleed at every point of urea injection, and even to start bleeding at the points from which blood had been withdrawn previously. Even in the most successful experiments, the haematocrit reading fell drastically, and the blood became extremely fluid. How much of this was due to continuous seepage of blood outward, and how much, if any, was due to an inward diffusion of water through the gills, remains obscure.

The greatest increase in plasma urea-N level obtained by urea injections was from 870 mgm.% before the injection to 1230 mgm.% afterward. The highest level obtained was 1550 mgm.%, a rise from a pre-injection level of 1240 mgm.%. These new levels were well below the maximum found occurring naturally. In these urea-injected animals there was no demonstration of a definite tubular maximum, but instead the reabsorption of urea from each cubic centimeter of filtrate was markedly depressed as compared with the uninjected animals. In preliminary results reported earlier (Kempton, 1948) the rate of reabsorption after injection was of the order of magnitude to be expected at the urea level prevailing before the injection. However, more data indicate that this was fortuitous, the rate of urea reabsorption not being "set" in relation to the pre-injection urea level.

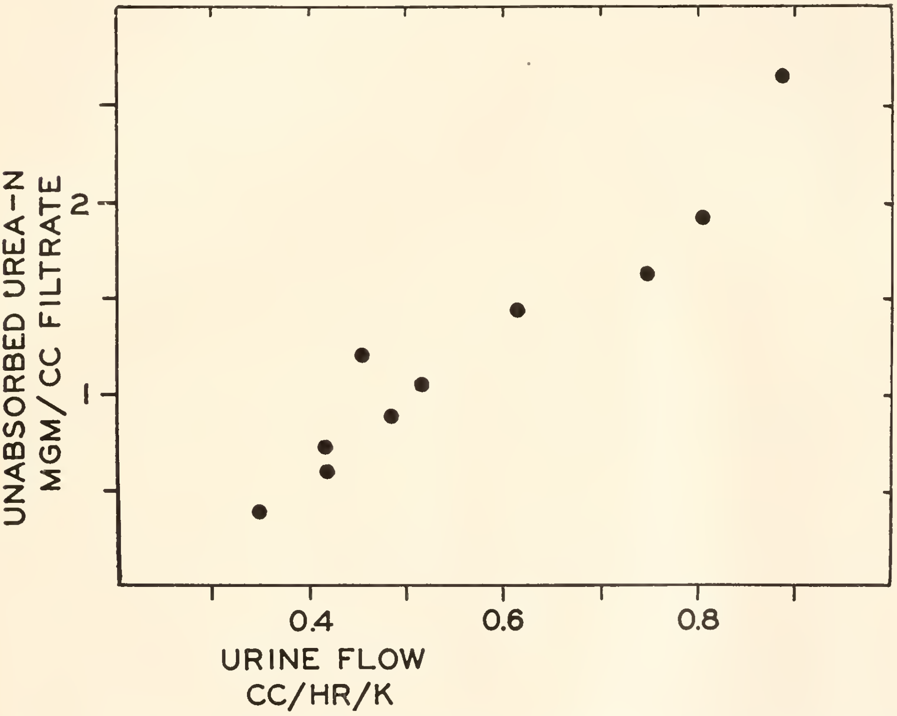


FIGURE 3. Variation in the unabsorbed urea in relation to the rate of urine flow. Each point represents the average of 10 collection periods.

This depression of urea reabsorption is probably without significance in relation to the reabsorptive process, because glucose and water reabsorption were similarly depressed. A comparison is given in Table I, in which the averages of the urea

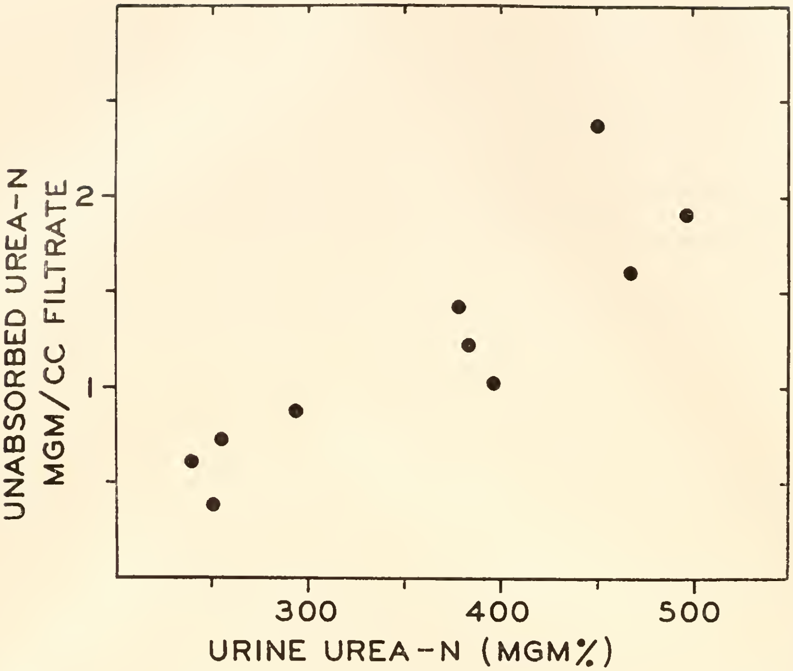


FIGURE 4. Variation in the unabsorbed urea in relation to the concentration of urea nitrogen in the urine. Each point represents the average of 10 collection periods.

TABLE I

*Comparison of averages of urea-injected animals with normal ones at the pre-injection and post-injection levels*

| | I | II | III |
|---|---|---|---|
| Plasma urea-N, mgm. % | 898 | 1196 | 1122 |
| Urine urea-N, mgm. % | 396 | 1083 | 306 |
| Filtration rate, cc./hr./k. | 2.38 | 1.64 | 1.68 |
| Urine rate, cc./hr./k. | 0.66 | 0.83 | 0.69 |
| $H_2O$ reabsorbed, cc./hr./k. | 1.69 | 0.80 | 1.00 |
| Urea-N reabsorbed, mgm./hr./k. | 17.6 | 10.6 | 16.6 |
| Glucose reabsorbed, mgm./hr./k. | 4.5 | 2.1 | 3.4 |
| Reabsorption per cc. of filtrate: | | | |
| $H_2O$ (cc.) | 0.72 | 0.47 | 0.53 |
| Urea-N (mgm.) | 7.76 | 6.04 | 9.75 |
| Glucose (mgm.) | 2.01 | 1.31 | 2.22 |

Column I. Group of 10 normal animals closest to the average plasma level of the injected animals previous to their injection.

Column II. Average of 9 collection periods from 5 animals injected with copious amounts of urea.

Column III. Group of 5 normal animals nearest to the average plasma level attained after the urea injections were made. More animals could not be used in this group because of the smaller numbers available at this relatively high urea level.

injected animals are compared on the one hand with an uninjected group selected to have a plasma level close to those prevailing before the urea injections were made, and on the other hand with a group of animals whose average plasma level compared with the experimental animals after the massive urea injections. If the kidney were "set" in its behavior by the pre-injection level, the second column of the table should correspond with the first; if the kidney behaved toward the injected urea as it does toward the endogenous urea there should be a correspondence between the second and third columns. Actually neither relation appears, but there is indication of a general depression of renal function as shown by the filtration rate, water reabsorption, urea reabsorption and glucose reabsorption.

TABLE II

*Data from 110 collection periods from smooth dogfish in good physical condition which had received inulin injections but no urea, based on data of summers of 1948 and 1950*

| | Minimum | Average | Maximum |
|---|---|---|---|
| Urine flow, cc./hr./k. | 0.11 | 0.63 | 1.72 |
| Filtration rate, cc./hr./k. | 0.53 | 1.82 | 3.51 |
| Urea clearance, cc./hr./k. | 0.05 | 0.24 | 0.81 |
| Glucose clearance*, cc./hr./k. | 0.03 | 0.16 | 0.29 |
| Inulin U/P ratio | 1.10 | 3.28 | 7.07 |
| Urea U/P ratio | 0.13 | 0.39 | 0.74 |
| Plasma urea-N, mgm. % | 730 | 1014 | 2140 |
| Urine urea-N, mgm. % | 120 | 373 | 603 |
| Plasma glucose,* mgm. % | 129 | 253 | 529 |
| Urine glucose,* mgm. % | 13 | 54 | 112 |
| Per cent reabsorption: | | | |
| $H_2O$ | 9 | 66 | 86 |
| Urea-N | 70 | 87 | 99.5 |
| Glucose* | 86 | 92 | 99.0 |
| Reabsorption per cc. of filtrate: | | | |
| $H_2O$ (cc.) | 0.09 | 0.65 | 0.86 |
| Urea-N (mgm.) | 5.2 | 8.9 | 18.5 |
| Glucose (mgm.)* | 1.2 | 2.3 | 3.5 |

* For glucose, only 51 collection periods are represented.

Since there is a paucity of data in the literature, it seems worthwhile to present some of the routine values obtained in these experiments, even though their significance for the present study is incidental. These are summarized in Table II. The values were obtained from animals which were used for a total of 110 collection periods during the summers of 1948 and 1950. There is no available explanation for the fact that the rates of urine flow obtained in a very short series of experiments in the summer of 1947 (Kempton and Steckler, 1947) were of a different order of magnitude, reaching a maximum of 3.94 cc./hr./k. as contrasted to 1.72 in the other two summers. In other respects these earlier animals were entirely in accord with the findings in 1948 and 1950, but because of the extreme differences in urine flow, and the possibility that this was a technical artifact due to unskillful handling of the animals, the few 1947 data have been omitted. Also omitted are the animals to which urea was administered, and a few periods in which the fish appeared to be moribund. Twelve collection periods with one spiny dogfish, *Squalus acanthias,* in 1948 gave values which were entirely in line with those of the smooth dogfish.

## Discussion

The data indicate that the rate of urea reabsorption is geared, directly or indirectly, to the plasma urea level. The only constant value is the average amount of urea remaining in the tubules unabsorbed from each cubic centimeter of filtrate. This relationship prevails through a range of normally occurring plasma levels in which the maximum is triple the minimum.

The reason that a similar relationship does not appear between total urea reabsorption in terms of mgm./hr./k. and the plasma concentration seems to be clear. In such a calculation the amount of urea would depend upon both the concentration in the filtrate and the volume of the filtrate. As the latter changed so would the amount of urea reabsorbed. But since the reabsorption is geared to the amount remaining unabsorbed in each unit volume of filtrate, the variations in total filtration introduce a random element which eliminates any apparent relationship.

The rate of reabsorption from each cubic centimeter of filtrate is not altered appreciably by the amount of filtrate produced nor the total amount of urea filtered. If all glomeruli are functioning all the time, differences in rate of filtration should result in a different rate of flow along the tubule, which in turn should be mirrored in variations in rate of urea reabsorption. This effect should certainly be clear when, as in these data, the rate of filtration falls along a seven-fold range. However, changes in filtration rate due to varying numbers of functional glomeruli would not result in a change of rate of flow. The fact that there is no change in urea reabsorption which can be correlated with changes in filtration rate indicates that the varying filtration rate in these experiments was produced by a change in the number of functional glomeruli rather than by increased or decreased function of individual units.

The variations in unabsorbed urea can be explained on either of two bases. These lead to two diametrically opposed conclusions in relation to the site of urea reabsorption as compared with that of water reabsorption. Variations in rate of filtration do not affect the urea reabsorption, apparently because there is no change in rate of flow of the filtrate, which has been discussed above. However, changes in amount of water reabsorption are related inversely to the amount of unabsorbed urea. This would be consistent with the idea that the osmotic effect of the greater amount of urea retained in the tubule inhibits reabsorption of water and leads to greater urine flow and a higher concentration of urea in the urine. The facts equally well fit the explanation that with less reabsorption of water there is a more rapid flow of filtrate which results in increased amounts of unabsorbed urea and higher urine levels. The first explanation would place urea reabsorption earlier in the tubule than water reabsorption or at the same level; the second would place it after the site of water reabsorption. No choice between the alternatives can be made on the basis of the present data. It is hoped that further experiments will permit a definite choice.

The reabsorption of urea cannot be isosmotic. While at lower plasma levels enough water is reabsorbed to account for an isosmotic reabsorption of all the urea and possibly the glucose and chloride as well, at higher plasma levels only a small fraction of the urea could be absorbed as an isosmotic solution, even if all the reabsorbed water were assigned to this purpose. This therefore leaves open the possibility that the controlling factor in urea reabsorption is a constant concentration of urea remaining in the tubules, possibly through the intervention of osmotic relationships. It is perhaps more likely that we are dealing with an enzyme system which functions rapidly only when a certain concentration of urea is present in the tubules.

## Summary and Conclusions

1. Water, urea and glucose reabsorption have been studied simultaneously using the inulin clearance method.
2. Variations in filtration rate appear to be due to changes in the number of functional glomeruli, rather than to changes in the rate of function of individual units.
3. Glucose reabsorption is not correlated in any way with that of urea.
4. The main factor controlling the urea reabsorption is the concentration of urea occurring normally in the plasma. Attempts to increase the reabsorption of urea by raising the plasma level result in a depression of reabsorption not only of urea but of glucose and water as well. To some extent this reduced water reabsorption is offset by a decreased filtration rate, with the result that only a very moderate diuresis ensues.
5. The percentage of the filtered urea which is reabsorbed varies with the concentration in the plasma, ranging from 70% to 99.5%, comparing quite favorably with the reabsorption of glucose.
6. On the average, the actual amount of unabsorbed urea left in each unit volume of filtrate is quite constant over a wide range of normally occurring urea levels.
7. Variations in the amount of unabsorbed urea are correlated in a presumably causal fashion with the reabsorption of water, the rate of urine flow and the concentration of urea in the urine.
8. Choice cannot be made at this time between alternate explanations of the data which would localize the site of urea reabsorption in relation to the site of water reabsorption.
9. The data show clearly that the reabsorption of urea cannot be an isosmotic one.

## LITERATURE CITED

Baldwin, Ernest, 1937. An introduction to comparative biochemistry. The University Press, Cambridge.

Clark, R. W., and H. W. Smith, 1932. Absorption and excretion of water and salts by the elasmobranch fishes. III. The use of xylose as a measure of the glomerular filtration in *Squalus acanthias*. *J. Cell. Comp. Physiol.*, **1**: 131–143.

Denis, W., 1922. The non-protein constituents in the blood of marine fish. *J. Biol. Chem.*, **54**: 693–700.

Hubbard, Roger S., and Ted A. Loomis, 1942. The determination of inulin. *J. Biol. Chem.*, **145**: 641–645.

Kempton, Rudolf T., 1943. Studies on the elasmobranch kidney. I. The structure of the renal tubule of the spiny dogfish (*Squalus acanthias*). *J. Morph.*, **73**: 246–263.

Kempton, Rudolf T., 1948. Urea excretion in the smooth dogfish. *Biol. Bull.*, **95**: 253–254.

Kempton, Rudolf T., and Burton Steckler, 1947. Urea excretion in the smooth dogfish. *Biol. Bull.*, **93**: 197.

Koch, Frederick C., and Martin E. Hanke, 1948. Practical methods in biochemistry. Williams and Wilkins, Baltimore.

Marshall, E. K., Jr., 1930. The comparative physiology of the kidney in relation to theories of renal secretion. *Physiol. Rev.,* **14**: 133–159.

Marshall, E. K., Jr., 1932. The secretion of urea in the frog. *J. Cell. Comp. Physiol.,* **2**: 349–353.

Nelson, Norton, 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.,* **153**: 375–380.

Shannon, J. A., 1936. Glomerular filtration and urea excretion in relation to urine flow in the dog. *Amer. J. Physiol.,* **117**: 206–225.

Smith, Homer W., 1931. The absorption and excretion of water and salts by the elasmobranch fishes. II. Marine elasmobranchs. *Amer. J. Physiol.,* **98**: 296–310.

Smith, Homer W., 1936. The retention and physiological role of urea in the Elasmobranchii. *Biol. Rev.,* **11**: 49–82.

Smith, Homer W., 1939. Studies in the physiology of the kidney. Porter Lectures, series IX, Univ. of Kansas Press, Lawrence, Kansas.

Walker, Arthur M., and Charles L. Hudson, 1937. The role of the tubule in the excretion of urea by the amphibian kidney. *Amer. J. Physiol.,* **118**: 153–166.

# THE CYTOCHEMICAL STAINING AND MEASUREMENT OF PROTEIN WITH MERCURIC BROMPHENOL BLUE[1]

DANIEL MAZIA, PHILIP A. BREWER AND MAX ALFERT

*Department of Zoology, University of California, Berkeley 4, California*

Various techniques have been adapted from protein chemistry to the cytological demonstration of proteins (*e.g.*, ninhydrin reaction, Mazia and Jaeger, 1939; Millon reaction, Pollister and Ris, 1947; Sakaguchi reaction, Thomas, 1946; Serra, 1946) but none has found wide use for both the resolution of morphological detail in terms of protein distribution and the measurement of relative protein concentrations. Danielli (1948) has presented an incisive summary of the problem and has proposed some ingenious new approaches, but these have not yet found common application.

The development of chromatographic and electrophoretic techniques which require identification of spots on filter paper has given new impetus to the study of color reactions of amino acids and proteins, and it is inevitable that the experience which is developing rapidly in this field will be carried over to the field of biological staining. Durrum (1950) devised the mercuric bromphenol blue reagent which has been widely adopted for the detection of protein spots, and the reaction has been studied in some detail by Kunkel and Tiselius (1951) and Geschwind and Li (1952). The present study deals with the application of the mercuric bromphenol blue procedure to cytological material. Its value as a simple staining technique, its capacity for bringing out in good contrast certain structures that often do not stain well by other procedures, and its specificity and applicability to the photometric estimation of proteins in cytological preparations will be considered.

## EXPERIMENTAL

1. *Preparation of tissue.* Common cytological fixatives, such as Carnoy's, Baker's formalin, Schaudinn's and Bouin's solutions, have been employed in this study. Of all fixatives tested, only those containing osmium interfere with this reaction. The alcoholic mercury-bromphenol blue reagent (Hg-BPB) as described by Durrum would in itself have the properties of a fixative so that theoretically fixation and staining could be carried out in one step. We have found in practice that fixation of blocks of tissue proceeds too slowly in this solution, and the slow penetration sets up artifacts in protein distribution due to the transport of unprecipitated protein within the cells by the flowing fixative. However, we have found the Hg-BPB solution to be satisfactory in the fixation of cell smears and of cilia (Fig. 1). Presumably it would be adapted to other surface structures.

---

[1] This work has been assisted by grants from the American Cancer Society, recommended by the Committee on Growth, National Research Council, and from the University of California Cancer Research Coordinating Committee.

It is necessary to work with quite thin preparations because the staining by this method is so intense in most cases. Sections of the order of 5 microns or less are recommended.

2. *Staining.* Staining may be carried out either in the alcoholic Hg-BPB solution described by Durrum or the aqueous solution used by Kunkel and Tiselius. In both cases the solution we have used contains 10 grams of $HgCl_2$ and 100 milligrams of bromphenol blue per 100 ml. The alcoholic solution is made up in 95 per cent ethanol. The preparations are immersed in the dye solution for 15 minutes. A longer period does not increase the intensity of staining. The preparations are then washed for 20 minutes in 0.5 per cent acetic acid as described by Kunkel and Tiselius. This solution removes excess dye but does not remove the dye that is presumably bound chemically, even after 5 hours of washing. Washing in water for 15 minutes is perfectly suitable for cytological purposes, but there is no sharp end point; with prolonged treatment with water more and more dye is removed. After the acid wash, the sections are immersed in water or buffer of pH 6–7 for three minutes to convert the dye to its blue alkaline form. Treatment with ammonia

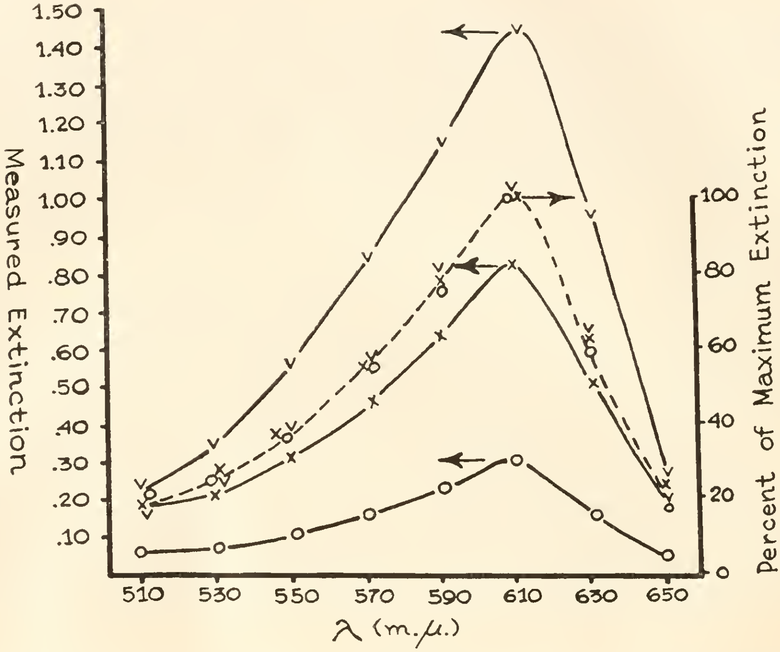


FIGURE 1. The validity of Beer's Law for the comparison of the Hg-BPB staining of objects of different optical density. Complete absorption spectra are given for the nucleolus of a sea urchin (*Strongylocentrotus purpuratus*) oocyte (—v—), cytoplasm of a rat intestinal mucosa cell (—x—) and cytoplasm of a mature sea urchin egg (—o—). The data on the egg cytoplasm are not comparable to Tables I and II since a thinner region was chosen in order to obtain a curve in a lower extinction range. In all three cases shown, a region 3 microns in diameter, producing a 3 mm. spot at the phototube level, was measured. Slit width: 2 mm.

vapor, as recommended for filter paper strips, is not suitable as it produces serious distortion of morphological detail.

These three steps constitute the whole procedure and yield reproducible results. All other standard manipulations appropriate to making temporary or permanent mounts may be carried out without apparent effects on the staining of the cells, except that prolonged soaking in dilute ethanol during dehydration may lead to some loss of dye.

## RESULTS

1. *Qualitative.* A variety of materials, plant and animal, has been studied. The procedure described is believed to be of general applicability as a cytological and histological technique. In the material we have studied, there was no known structure that was not brought out in good contrast to its background by the technique, and this is not at all surprising since it is difficult to imagine the existence of a cell structure without some concomitant differentiation in protein composition and concentration. Each of the structures selected for purposes of illustration may, of course, be stained by other means, but for the most part different techniques would be required in different cases. Fixatives and stain solutions employed are designated in the legends to the photographs.

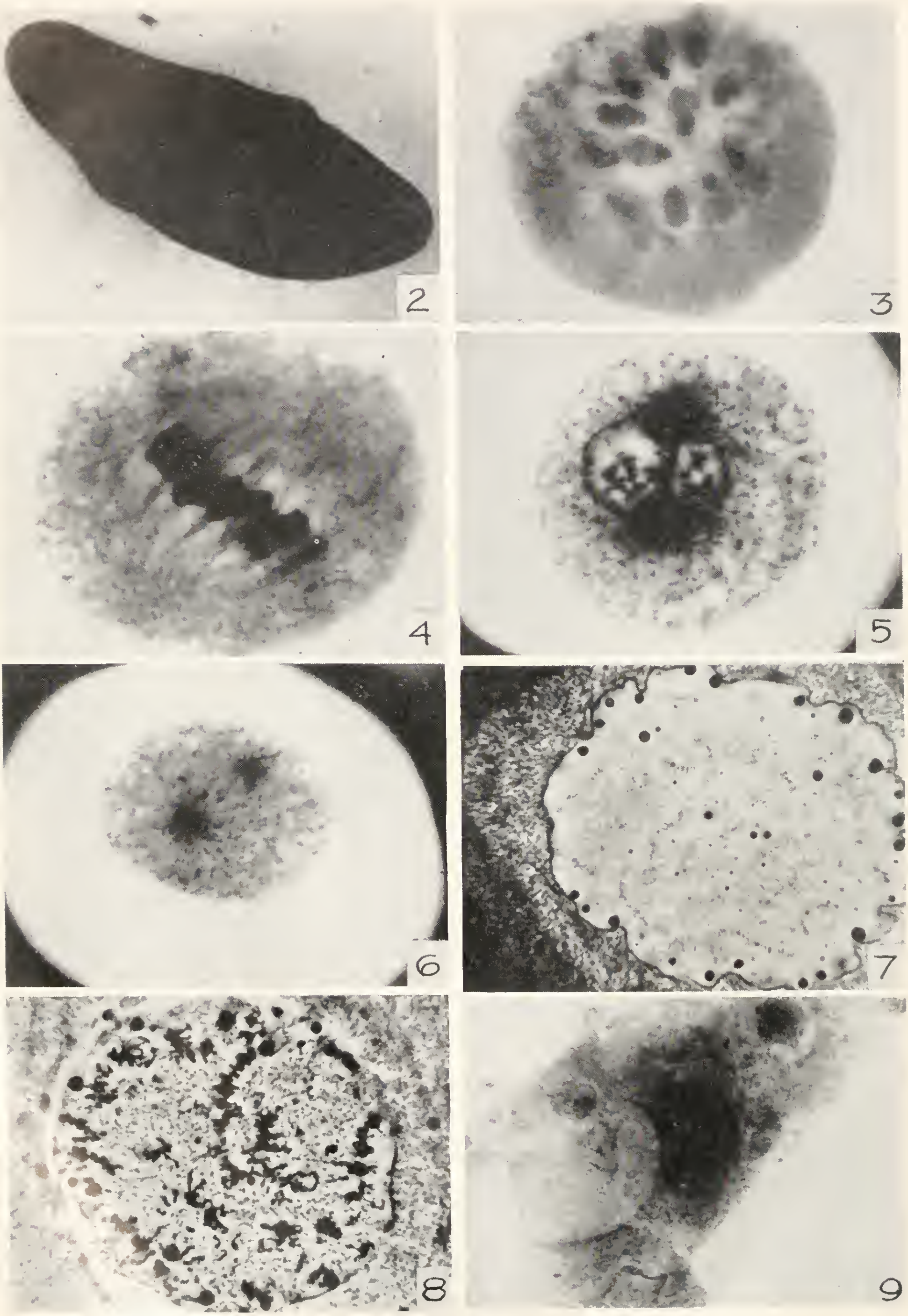
(1) *Cilia.* Figure 2 shows a *Paramecium* fixed and stained directly in the alcoholic Hg-BPB solution. This cell is too thick for the observation of internal detail but the cilia, which ordinarily require special staining techniques, appear dark and distinct. 3

(2) *Mitotic figures.* Figures 3 and 4 show microspore divisions in *Lilium longiflorum.* Figure 2 is a metaphase plate and is of special interest because the regions of attachment to the spindle fibers seem to be distinguished from the other regions of the chromosomes by their greater dye-binding capacity. Figure 3 is a later stage in equatorial view, showing strong staining and apparent structural detail of the spindle components. Each chromosome seems to be connected to the polar cytoplasm by a bundle of fibrils. 4

Figure 5 shows an early stage in the first division of an egg of *Anascaris equorum.* The material of the "achromatic figure" (by this technique the most densely staining structure in the cell) is seen in the form of two asters connected by a mass of staining material lying between the adjacent pronuclei. In Figure 6, an equatorial view of an early anaphase in the first cleavage of the same form, the complete mitotic figure, with asters, spindle and two chromosomes, is seen.

Dr. Sajiro Makino (personal communication) has applied this technique to the demonstration of meiotic figures in orthopteran and hemipteran testes with satisfactory results.

(3) *Lamp brush chromosomes.* The giant "lamp brush" chromosomes found in the oocytes of amphibian and other lower vertebrates are difficult to stain by most methods because the nucleic acid is rather dispersed. The Hg-BPB technique brings out their structure clearly. Figure 7 shows an oocyte of the frog *Rana pipiens* and Figure 8 an oocyte of the urodele *Batrachoceps attenuatus.* In the latter case, the chromosomes are more condensed and therefore more conspicuous. The frog chromosomes are highly extended, but the stain differentiates their characteristic organization as a thread with long lateral projections or loops rather well.



FIGURES 2–9.

This picture is interesting because it corresponds so well to that obtained, in more favorable species, in living nuclei (Duryee, 1950).

(4) *Amoeba.* In our experience, the nucleus of *Amoeba proteus* is brought out only in very poor contrast with nucleic acid stains. The concentration of nucleic acids seems to be relatively low in this large nucleus. Figure 9 shows an individual *Amoeba proteus* stained by the Hg-BPB technique, showing the nucleus to be a structure where the protein concentration is much higher than that obtaining in the cytoplasm.

These preparations, as well as others we have obtained, contain a number of features of considerable cytological interest, which will not be discussed at this time. Some will be evident from inspection of the figures. The crispness and clarity of the results have been noteworthy if only because they are obtained by a rapid method that requires no modification from one type of material to another.

2. *Quantitative.* Preliminary to a study of the specificity of the Hg-BPB stain, a series of measurements has been made to determine whether the Beer and Lambert laws apply to material stained by this method. The apparatus and technique were those previously described by Alfert (1950), except that a Beckmann Model B spectrophotometer was used as a light source.

The absorption maximum of the dye-protein complex in tissue is 610 mμ. The test of the applicability of Beer's Law was carried out in two ways. First, extinction measurements at various wave-lengths were made on three different regions within the same cell: the cytoplasm, nucleus and nucleolus of oocytes of the sea urchin *Strongylocentrotus purpuratus.* In Table I the absolute and relative extinction values are given for four wave-lengths. The measurements given in the table were made on the same cell, cut at 5 microns. Therefore the extinction values for different regions are comparable. The "relative" values were calculated by letting the measured extinctions at 610 mμ equal 100. It is seen that while the intensity of staining varies from one region to another, the relative extinctions are nearly the same at different wave-lengths, as required if Beer's Law is valid for the situation. Figure 1 leads to the same conclusion by a somewhat different approach. Complete absorption curves were taken for three materials—

---

FIGURES 2–9.

FIGURE 2. *Paramecium caudatum.* Fixed and stained in aqueous Hg-BPB. Whole mount. × 330.

FIGURE 3. *Lilium longiflorum.* Microspore division. Polar view of metaphase plate. Fixation: Carnoy's solution. Sectioned at 8 μ. Stain: aqueous Hg-BPB. × 1240.

FIGURE 4. *Lilium longiflorum.* Microspore division. Equatorial view of anaphase. Fixation, sectioning, and staining as in Figure 3. × 1330.

FIGURE 5. Egg of *Anascaris equorum.* Stage of close approach of pronuclei. Fixation: Carnoy's solution. Section at 8 μ. Stain: aqueous Hg-BPB. × 970.

FIGURE 6. Egg of *Anascaris equorum.* Equatorial view, first cleavage division. Note staining of asters, spindle, and the two chromosomes. Prepared as in Figure 5. × 830.

FIGURE 7. Ovary of *Rana pipiens.* "Germinal vesicle" of oocyte. Note staining of nucleoli and fine "lamp brush" chromosomes. Fixation: Bouin's solution. Sectioned at 10 μ. Stain: aqueous Hg-BPB. × 300.

FIGURE 8. Ovary of *Batrachoceps attenuatus.* "Germinal vesicle." "Lamp brush" chromosomes seen in longitudinal and transverse section. Note structure of projecting loops. Fixation: Bouin's solution. Sectioned at 10 μ. × 350.

FIGURE 9. Part of an *Amoeba proteus,* showing intense protein-staining of nucleus. Fixation: Carnoy's solution. Squash preparation. Stain: aqueous Hg-BPB. × 430.

TABLE I

*The validity of Beer's Law for the Hg-BPB staining of cell contents (sea urchin oocyte)*

| Wave-length | Extinction | | | | | |
|---|---|---|---|---|---|---|
| | Cytoplasm | | Nucleus ("sap") | | Nucleolus | |
| | Measured | Relative | Measured | Relative | Measured | Relative |
| 530 | 0.187 | 33 | 0.194 | 36 | 0.265 | 27 |
| 570 | 0.314 | 55 | 0.314 | 59 | 0.606 | 61 |
| 610 | 0.568 | 100 | 0.537 | 100 | 1.00 | 100 |
| 650 | 0.068 | 12 | 0.089 | 17 | 0.052 | 15 |

the sea urchin oocyte nucleolus, the cytoplasm of mucosa cells of rat intestine, and the cytoplasm of the mature sea urchin egg. The extinction values at 610 mμ range from 0.3 to 1.44. The absolute extinction curves are evidently parallel, but the close parallelism is best shown in curve 4, where the relative extinctions for all three materials are plotted on the same scale by setting the maximum value as 100. Over such a wide range of extinction values, deviations from Beer's Law through recognized mechanisms (Sandell, 1944) would be expressed as changes in the absorption curves.

A rather elegant means of testing the applicability of Lambert's Law was provided by sectioned sea urchin egg populations, where we frequently encountered cases in which sections of two cells slipped and partially overlapped. It was thus possible to compare the absorption by single sections of two eggs with the absorption of the regions where these were superimposed. The results are given in Table II where it is seen that the extinction values are additive to a satisfactory degree. The several tests listed in Table II were made on different pairs of cell-sections. Regions 4 microns in diameter were measured, giving a 4 mm. spot at the level of the phototube.

In visual inspections of slides stained by this method, it often appeared that the most intensely stained preparations also seemed to have a more reddish hue. At

TABLE II

*Tests of the validity of Lambert's Law for measurement of Hg-BPB staining of sea urchin egg cytoplasm. Measurements made on adjacent 5 micron sections (a and b) and on region where they overlap (a + b)*

| Test No. | Extinction at 610 mμ | | | |
|---|---|---|---|---|
| | Section a | Section b | Measured a + b | Deviation from calculated a + b |
| 1 | 0.602 | 0.613 | 1.202 | −1% |
| 2 | 0.612 | 0.777 | 1.284 | −8% |
| 3 | 0.614 | 0.665 | 1.362 | +6.5% |
| 4 | 0.754 | 0.599 | 1.237 | −9% |
| 5 | 0.612 | 0.615 | 1.236 | +1% |

first this seemed to suggest the complication of metachromasia or some related effect. However, the photometric measurements revealed no difference between the bluer and the redder preparations. The reddish appearance of densely stained material may be explained in terms of the spectral sensitivity of the human eye, but is irrelevant to the present discussion since it has no influence on objective measurements.

The demonstration of the validity of photometric laws does not in itself imply that we are measuring the concentration of any normal constituents of the cell. In discussing this common misconception in relation to Beer's Law, Sandell (1944, p. 61) says, ". . . this law simply states that the extinction must be proportional to the concentration of the colored substance; it does not state that the extinction must be proportional to the analytical concentration of the constituent which forms the colored substance." Such an assurance can only come from chemical investigation of the staining reaction, as described below. But the above data do indicate that, insofar as we can interpret the dye-binding reaction, the microphotometric measurements will accurately follow its stoichiometry.

Since the Millon reaction has proved to be useful in photometric studies on cell proteins, it is of interest to compare it with the Hg-BPB reaction. A disadvantage of the Millon technique is that the compound formed absorbs so weakly in the visible. While satisfactory extinctions may be obtained in the near-ultraviolet region, the structures to be measured are difficult to locate visually. We have compared the extinction values of sea urchin egg cytoplasm stained by the Millon procedure (measured at 355 mμ) and by the BPB procedure (measured at 610 mμ). The average of 10 measurements by the BPB procedure for a given series of sections was 0.646. The Millon reaction gave an average extinction of 0.121. Thus the BPB reaction provides greater optical density with a given concentration of material as well as an absorption maximum in a more convenient range.

TABLE III

*The binding of Hg-BPB by various substances on filter paper*

| Substance | Moles dye bound per mg. |
|---|---|
| Starch | 0 |
| Glycogen | 0 |
| DNA | $5.9 \times 10^{-9}$ |
| Cholesterol | $5.4 \times 10^{-9}$ |
| Bovine serum albumin (cryst.) | $1.20 \times 10^{-6}$ |
| Chymotrypsin (cryst.) | $1.06 \times 10^{-6}$ |
| Pepsin (cryst.) | $1.03 \times 10^{-6}$ |
| Histone (pH 4) | $1.40 \times 10^{-6}$ |

3. *Specificity and quantitative significance.* Few tests for protein actually measure the mass of protein. What is usually determined is some chemical group that is characteristic of proteins. The tests give information concerning the amount of protein present only insofar as one has independent information as to the distribution of the particular group or groups in the protein under observation.

Obviously, the first consideration is the qualitative specificity of the BPB reaction; whether the dye is combined by non-protein constituents of the cell. We have tested, on filter paper, the dye-binding by a series of substances that might confuse the reaction. The following table (Table III) indicates that the dye-binding by the non-protein materials tested is negligible compared with that of protein. The procedure was exactly as described above, carried out on filter-paper spots. The amount of dye was estimated after extraction of the paper with ammoniacal acetone, the standard being a known solution of dye in the same solvent.

The question of the BPB reaction of amino acids and polypeptides has been studied in detail by Geschwind and Li (1952). From their work it appears that many free amino acids and peptides would bind dye, but the dye complexes—with the exception of those involving histidine and possibly cysteine—would be dissolved away during the washing of the preparation.

From the cytological standpoint, one is most interested in the possibility of distinguishing protein in structures containing nucleic acid. We therefore studied the BPB reaction in cells from which nucleic acids had been removed by extraction with hot 5 per cent trichloroacetic acid for 15 minutes. No decrease in the BPB staining is observed. In fact, certain structures, especially the chromosomes, now stained more intensely, as would be anticipated if the nucleic acid were blocking potential dye-binding groups. The fact that the intensity of chromosome staining increased is consistent with the view that many of the basic groups of nuclear proteins are bound to the acid groups of nucleic acid.

Kunkel and Tiselius (1951) have shown that the amount of dye bound is proportional to the amount of protein over a wide range. They found, however, a 30 per cent difference in the dye-binding per milligram of albumin and gamma globulin. We have tested a number of pure proteins and mixtures containing proteins (tissue homogenates) on filter paper and have consistently observed the same linearity and variability. Our highest and lowest values for dye bound per milligram protein differ by about 40 per cent. Our procedure differed from that of Kunkel and Tiselius only in that the dye was extracted from the paper by ammoniacal acetone. On the average, one milligram of protein combined about $10^{-6}$ moles of dye. Taking 120 as a representative weight of an amino acid residue, this would mean that one molecule of dye is bound for, approximately, every 10 amino acid residues present.

In view of this variability the method will be applicable to the exact comparison of total protein concentrations only where the qualitative differences are expected to be small. It will be suitable for the approximation of large differences, since the variation of dye-binding among various proteins studied is by no means one of order of magnitude.

The variability is true of most protein methods, which generally involve the measurement of certain characteristic groups in proteins. It does not seem likely, from the results reported, that the method is specific for a single group. As mentioned, we have found that in a variety of proteins the ratio of moles of dye bound to amino acid residues present is roughly 0.1. Examining the composition of the proteins, we find no single type of amino acid that accounts for 10 per cent of those present.

If we examine those groups which could potentially enter the reaction, these seem to be the $NH_2$ groups of basic proteins, which might combine by direct salt formation, and the SH groups, aromatic groups and free COOH group which might be coupled to the dye through the mercury.

The experiments showing enhanced staining of chromosomes after extractions with trichloroacetic acid indicate that the basic groups of basic proteins may contribute to the dye-binding. This would be a simple case of acid-staining, in the microscopists' terminology, and would not be expected to involve combination with mercury. A number of experiments, both with pure proteins on filter paper and with tissues, suggests that this direct combination of the dye with basic groups is, in fact, largely limited to basic proteins, but that a mechanism involving coupling through Hg is responsible for the major part of the staining of tissues and of ordinary proteins. In the work with pure proteins on filter paper it was shown that spots of protamine stained equally well whether the Hg was present or absent. On the other hand, ovalbumin stained only in the presence of Hg; when Hg was omitted, there was a loose attachment of dye which readily washed out in the standard procedure. Since the function of the Hg might merely be that of keeping the protein on the paper, we returned the albumin-containing papers, which had been washed free of dye after treatment with the Hg-free reagent, to the normal Hg-containing dye solution. The spots now stained normally and the dye could not be washed out. Therefore the Hg plays a role in coupling the dye to the protein. In these experiments, the protein spots were fixed with 3 : 1 alcohol-acetic acid and dehydrated with alcohol.

Exactly the same relationship held for tissues. When these were treated with a dye solution without Hg, they seemed to take up a considerable amount of dye, but most of it was removed on washing. Under the microscope, a typical picture of acid-staining was observed. It is suggested that a comparison of staining with and without Hg, and before and after the extraction of nucleic acids, might provide a basis for estimating the relative amount of basic protein and the extent to which the basic groups were free, but this has not been investigated in detail.

The groups which might combine the dye through Hg, the SH, aromatic and free COOH groups, might be differentiated by blocking with appropriate group-reagents, and it is proposed to investigate this possibility.

## Discussion

For the cytochemist who is interested in estimating the total concentration of protein, rather than some characteristic side chain, the ideal method would be one in which each amino acid or peptide linkage would produce an equivalent light absorption. Such a method would be provided by the biuret reaction, for instance, though the extreme alkaline conditions required render this unsuitable for cytological purposes. The next best approximation is a method which will register such a variety of groups as to minimize, by a statistical "averaging-out," the consequences of compositional differences among proteins. The Hg-BPB method would seem to have this advantage. Inspection of tables of protein composition shows that the aggregate percentage of the amino acids potentially reacting in this method varies less from protein to protein than the percentage of any one amino acid.

The method has the further advantages that there is no class of proteins that will not be stained significantly and that, insofar as the composition of the test-object is known, the quantitative differences in the dye-binding by different proteins may be assessed accurately by parallel studies on filter paper. The studies that have already been made by Kunkel and Tiselius and by ourselves show that proteins and protein mixtures may readily be analyzed by simple filter-paper techniques with excellent correlation between the amount of protein present and the amount of dye bound. The variation that we have discussed previously seems to be a genuine index of qualitative differences among proteins, and subject to analysis.

The fact that the dye is combined to different groups and by at least two different mechanisms offers certain interesting possibilities. For instance, the staining without Hg appears to be a typical case of acid staining, which has been used by others (*e.g.*, Schrader and Leuchtenberger, 1950) as a measure of basic proteins. If bromphenol blue is used, and the staining in the presence and absence of Hg is compared, one has a system of comparison of basic protein to total protein in which at least the photometric units (extinction, optical density) can be compared directly in terms of the number of dye-binding groups. This would not be the case if different dyes were used for the two classes of proteins. When it becomes possible to sort out the various groups that combine the dye through Hg, the same advantage will obtain. In this way, the Hg-BPB method should be a useful complement to those methods which are selective for one group, such as the Millon method for phenolic residues and the methods specific for SH groups (Tahmisian and Brues, 1951; Bennett, 1951).

## SUMMARY

1. The mercuric bromphenol blue reaction as used for development of protein spots on filter paper has been found to be applicable to the cytological staining of proteins.

2. The optimum procedure is identical in detail with that described by Kunkel and Tiselius for filter paper spots, except that a neutral aqueous solution is substituteed for ammonia vapor in the final color development.

3. The sharp and intense staining of protein permits good differentiation of structures often difficult to observe, such as cilia, spindle elements, regions of spindle fiber attachment to chromosomes and "lamp brush" chromosomes.

4. The procedure is specific for proteins and those peptides which are not removed in the washing procedure.

5. The preparations stained by this procedure follow the Beer and Lambert Laws in microspectrophotometric measurements. The absorption maximum is at 610 millimicrons.

6. Basic proteins bind the dye under the conditions of the method even when Hg is omitted. Other proteins bind the dye by coupling through Hg. As expected, structures containing basic proteins show enhanced staining after removal of the nucleic acid.

7. The number of groups in various proteins binding the dye in this procedure varies somewhat, but the average is about one dye-binding group per 10 amino acid residues.

## LITERATURE CITED

ALFERT, M., 1950. A cytochemical study of oogenesis and cleavage in the mouse. *J. Cell. Comp. Physiol.*, **36**: 381–409.

ALLFREY, V., H. STERN, A. E. MIRSKY AND H. SAETREN, 1952. The isolation of cell nuclei in non-aqueous media. *J. Gen. Physiol.*, **35**: 529–558.

BENNETT, H. S., 1951. The demonstration of thiol groups in certain tissues by means of a new colored sulfhydryl reagent. *Anat. Rec.*, **110**: 231–248.

DANIELLI, J. F., 1948. Studies on the cytochemistry of proteins. *Cold Spring Harbor Symp. Quant. Biol.*, **14**: 32–39.

Durrum, E. L., 1950. A microelectrophoretic and microionophoretic technique. *J. Amer. Chem. Soc.*, **72**: 2493–2498.

Duryee, W. R., 1950. Chromosomal physiology in relation to nuclear structure. *Ann. N. Y. Acad. Sci.*, **50**: 920–942.

Geschwind, I. R., and C. H. Li, 1952. The reaction of bromphenol blue with amino acids and peptides. *J. Amer. Chem. Soc.*, **74**: 834–835.

Kunkel, H. G., and A. Tiselius, 1951. Electrophoresis of proteins on filter paper. *J. Gen. Physiol.*, **35**: 89–118.

Mazia, D., and L. Jaeger, 1939. Nuclease action, protease action and histochemical tests on salivary chromosomes of Drosophila. *Proc. Nat. Acad. Sci.*, **25**: 456–461.

Pollister, A. H., and H. Ris, 1947. Nucleoprotein determinations in cytological preparations. *Cold Spring Harbor Symp. Quant. Biol.*, **12**: 147–157.

Sandell, E. B., 1944. Colorimetric determination of traces of metals. Interscience. New York.

Schrader, F., and C. Leuchtenberger, 1950. A cytochemical analysis of functional interrelations of various cell structures in *Arvelius albopunctatus* (de Geer). *Exp. Cell. Res.*, **1**: 421–452.

Serra, J. A., 1946. Histochemical tests for proteins and amino acids; the characterization of basic proteins. *Stain Technol.*, **21**: 5–18.

Tahmisian, T. N., and A. M. Brues, 1951. A new method for staining cells with cobalt and BAL. *Nuclear Science Abstr.*, **5**: 323.

Thomas, L. E., 1946. A histochemical test for arginine-rich proteins. *J. Cell. Comp. Physiol.*, **28**: 145–158.

# STUDIES IN THE DEVELOPMENT OF FROG HYBRIDS. IV. COMPETENCE OF GASTRULA ECTODERM IN ANDROGENETIC HYBRIDS

JOHN A. MOORE AND BETTY C. MOORE

*Departments of Zoology, Barnard College and Columbia University, New York, N. Y.*

Hybrid embryos obtained from the fertilization of *Rana pipiens* Schreber ova with sperm of *Rana sylvatica* Le Conte develop in a seemingly normal manner to the beginning of gastrulation (Moore, 1946). In the majority of cases the hybrids form a tiny dorsal lip and differentiate no further. They remain as arrested gastrulae for nearly a week and then cytolize. The failure of further differentiation of these hybrids has been found to be correlated with reduced competence of the presumptive epidermis (Moore, 1947) and reduced inductive ability of the dorsal lip (Moore, 1948) as compared with normal embryos.

In a further effort to dissociate the factors responsible for the cessation of development in the hybrids, a study has been made of androgenetic hybrids of *R. pipiens* ♀ × *R. sylvatica* ♂ obtained by removal of the maternal chromosomes. The development of these haploid hybrids tests the ability of the *sylvatica* nucleus to influence the development of the *pipiens* egg devoid of *pipiens* chromosomes. It must not be imagined, however, that the enucleated *pipiens* ovum is merely a passive substrate for a foreign sperm to mould. We shall be studying the effect of *sylvatica* genes on the egg cytoplasm that was organized in the ovary under the influence of *pipiens* genes. There is a considerable body of evidence to indicate that the development of the egg up to late blastula or early gastrula is already determined at the time it leaves the female (Moore, 1941). In the case of these androgenetic hybrids, therefore, we should not expect an effect of the *sylvatica* chromosomes during the cleavage and blastula stages.

## MATERIALS AND METHODS

Eggs were obtained from a *R. pipiens* by pituitary injections. Some were fertilized with *pipiens* sperm and others with *sylvatica* sperm. Using the method of Porter (1939), the maternal chromosomes were removed from a number of the eggs. The four classes of embryos obtained were normal *pipiens*, *pip* × *pip;* haploid *pipiens*, (*pip*) × *pip;* hybrids, *pip* × *syl;* and haploid hybrids, (*pip*) × *syl.*

The *pip* × *pip* embryos developed normally. The (*pip*) × *pip* embryos were identical with the diploid normals during the early stages. The first deviation from normality occurred during gastrulation, when there was a slight retardation in the rate of development. In later stages morphological abnormalities, characteristic of the haploid syndrome (Porter, 1939; Moore, 1950), appeared and the embryos died as edematous larvae.

The *pip* × *syl* hybrids developed normally to the beginning of gastrulation. Thereafter, development was completely abnormal (Moore, 1946). Wrinkles and furrows formed in the blastocoel roof, which later became smooth as the entire embryo swelled. Eventually the blastocoel roof appeared to collapse and the embryo was left as a much wrinkled gastrula. Cytolysis began when the *pip* × *pip* embryos were in stage 20 (gill circulation) or 21 (cornea transparent).

The (*pip*) × *syl* embryos developed normally until the late blastula stage, as has been noted previously by Ting (1951). Epiboly was not as extensive as in *pip* × *syl* and the dorsal lip never formed. A grayish region on one side of the embryo may have been an indication of where the dorsal lip would have appeared, if differentiation had proceeded further. The (*pip*) × *syl* embryos, like the diploid hybrids, became swollen and remained alive until the normal *pipiens* controls reached stage 21. Thus, at a temperature of 20°, they remained as arrested blastulae for approximately one week. During this period the haploid hybrids appeared perfectly healthy and did not exhibit the characteristic wrinkles, pits and furrows of the diploid hybrids, nor did they shrink in size before cytolysis.

Since diploid hybrids of *pip* × *syl* have a dorsal lip, and haploid hybrids of (*pip*) × *syl* do not, it is possible to be sure of using the desired embryos for transplantation. The few embryos in the (*pip*) × *syl* group which formed a dorsal lip were assumed to be diploid, due to failure of removal of the maternal chromosomes, and were not used in the transplantation experiments. The (*pip*) × *pip* early gastrulae can be definitely distinguished from the *pip* × *pip* embryos by the slight retardation in development.

## EXPERIMENTS

Competence of the gastrula ectoderm was tested by transplanting pieces of the blastocoel roof of an early gastrula to the pronephric region of older embryos, in a manner previously described (Moore, 1947). Such transplants, under the influence of the host cells, form neural tissue and other structures if competent, as Holtfreter (1933) has demonstrated. The host in all of the experiments was *Rana palustris* Le Conte. Embryos of this species are much lighter in color than *R. pipiens* embryos and, therefore, host and donor cells are readily distinguishable. This difference in pigmentation is apparent in histological preparations as well. The hosts were in stage 17 (tail-bud) at the time of transplantation. One donor contributed transplants to two hosts.

Fourteen transplants of (*pip*) × *syl* presumptive ectoderm were made and compared with similar transplants of 8 (*pip*) × *pip*, 10 *pip* × *syl* and 2 *pip* × *pip*. The (*pip*) × *pip* transplants served as the controls for haploidy, while the *pip* × *syl* experiments formed the chief basis of comparison. The behavior of *pip* × *syl* and *pip* × *pip* tissue has been studied extensively in previous experiments (Moore, 1947), so relatively few operations of this material were performed for the present experiments.

It was found that when the control *pip* × *pip* were early gastrulae, the (*pip*) × *pip* were slightly earlier gastrulae; the *pip* × *syl* showed pigment at the dorsal lip and no invagination; and the (*pip*) × *syl* were late blastulae. Since it was desirable to have the different transplants in the same relative stage of differentiation, transplants were made from *pip* × *pip*, (*pip*) × *pip* and *pip* × *syl* when they were in stage 10 (early gastrulae). Since the *pip* × *syl* are arrested at stage 10, and the formation of the dorsal lip is retarded, they were used when they had been in this stage for a short time. Control *pip* × *pip*, fertilized at the same time and kept under the same conditions, were in stage 11 (semi-circular blastopore). The (*pip*) × *syl* had to be used in stage 9 (late blastulae), as they never form a dorsal lip. Since their development was retarded at this time, they were used when the control *pip* × *pip* were in stage 12 (yolk plug).

In the transplants from the three control types, the *palustris* hosts were allowed to develop to stage 21. They were then fixed and studied in serial section. In the transplants involving (*pip*) × *syl* tissue, the hosts were fixed in stage 20, since some of the transplants were unhealthy at that stage.

## RESULTS

The 14 transplants of (*pip*) × *syl* ectoderm showed no differentiation whatsoever. The cells remained as large, late blastula cells. There was little variation among the 14 cases and Figures 1 and 2 may be taken as typical of the results. It was noticed that the donor cells stained much less intensely than the host cells with fast green.

The 10 *pip* × *syl* transplants showed slightly better differentiation than the haploid hybrid transplants. The cells were smaller in size than in the (*pip*) × *syl* experiments. A two-layered epidermis was formed with an underlying mass of tissue which could not be called either neural or neuroid. The degree of differentiation was similar to that shown in Moore (1947), Figure 8. This represents the lowest degree of differentiation that can be expected with this tissue.

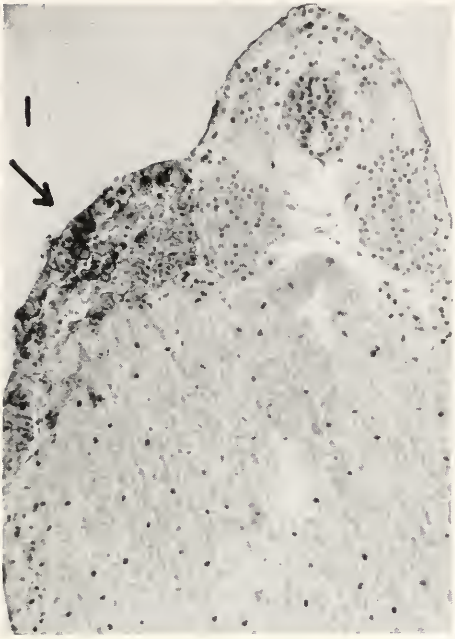
The 8 (*pip*) × *pip* transplants were distinctly better in their differentiation than either previously mentioned class. The transplants formed a two-layered epidermis with underlying neuroid masses. Figures 3 and 4 are typical.

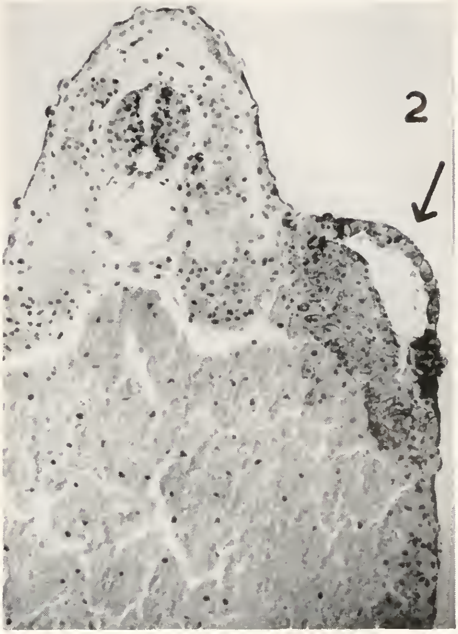
The two *pip* × *pip* transplants showed the greatest differentiation. As in accordance with earlier work (Moore, 1947), neural tissue was formed.

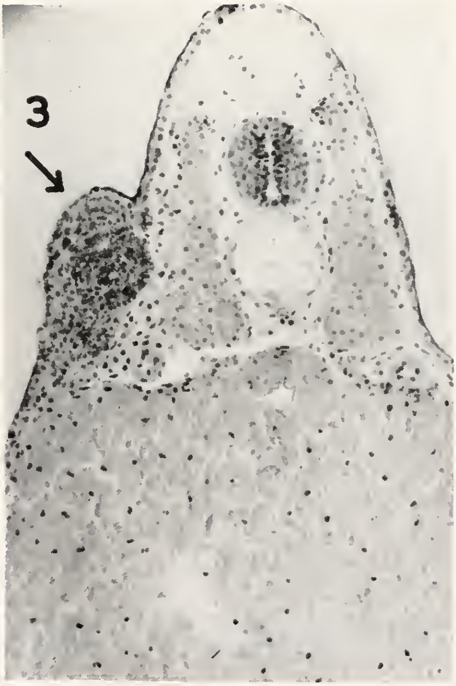
## DISCUSSION

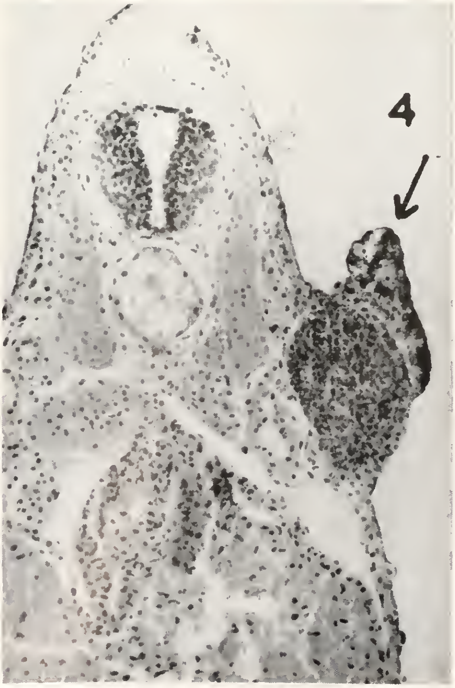
From the data described above, it is evident that the presumptive ectoderm of (*pip*) × *syl* is lacking in competence and, when transplanted, cannot differentiate any further than in the entire haploid hybrid embryo. This result throws some light on the factors involved in the cessation of development of the diploid hybrids. We may imagine that there are three main components in a *pipiens* ♀ × *sylvatica* ♂ hybrid, namely: *pipiens* cytoplasm, a haploid set of *pipiens* chromosomes and a haploid set of *sylvatica* chromosomes. When all three components are present, normal development ceases at the beginning of gastrulation. Since the combination of *pipiens* cytoplasm plus a haploid set of *pipiens* chromosomes alone can produce an embryo which reaches the larval stage of development, it is clear that the addition of a haploid set of *sylvatica* chromosomes is having an antagonistic effect on the functioning of the *pipiens* chromosomes, or of the *pipiens* cytoplasm or of both. Since the development of an embryo with *pipiens* cytoplasm and a haploid set of *sylvatica* chromosomes is even less after the removal of the haploid set of *pipiens* chromosomes, it is evident that there is an antagonistic effect between the *sylvatica* chromosomes and the *pipiens* cytoplasm. That this is a real antagonism has been demonstrated by the transplantation experiments, where no response can be elicited

PLATE I









FIGURES 1 AND 2. Development of androgenetic *pipiens* ♀ × *sylvatica* ♂ gastrula ectoderm in *Rana palustris*. The region of the donor tissue is indicated by an arrow.

FIGURES 3 AND 4. Development of androgenetic *pipiens* gastrula ectoderm in *Rana palustris*. The region of the donor tissue is indicated by an arrow.

in the haploid hybrid tissue, even though in a normal environment. From these experiments it is evident that the result of the antagonistic action between the *sylvatica* chromosomes and the *pipiens* cytoplasm is actually lessened by the presence of a set of *pipiens* chromosomes, since the development of the diploid hybrid is greater than that of the haploid hybrid, and transplanted ectoderm is capable of considerably greater differentiation (Moore, 1947).

A finding of considerable interest is the evidence of a gradation in competence of the presumptive ectoderm of the various types of embryos studied. The results indicate that diploid *pipiens* presumptive ectoderm is more competent than haploid *pipiens* presumptive ectoderm. The last is more competent than the diploid hybrid presumptive ectoderm which is more competent than the haploid hybrid presumptive ectoderm.

Although only two transplants of *pip* × *pip* tissue and 8 of (*pip*) × *pip* tissue were made, in a previous experiment (Moore, 1947) 18 transplants of *pip* × *pip* gastrula ectoderm were described. In each one of these 20 transplants, neural tissue was formed from the donor cells, whereas none of the 8 (*pip*) × *pip* transplants formed good neural tissue. Their response was at a distinctly lower level of differentiation which is customarily called neuroid. More recent experiments (unpublished data) have shown that out of 33 *pip* × *pip* transplants, 91% formed neuroid or neural tissue, but such a response was shown by only 44% of 32 (*pip*) × *pip* transplants.

Similar testing of the competence of haploid tissue has not been studied by other workers. In all probability the degree of competence of such tissue will vary from species to species, even as the development of whole haploid embryos varies. An androgenetic *Triton taeniatus* has reached metamorphosis (Baltzer, 1922; Fankhauser, 1938) and obviously such haploid tissue is competent and capable of normal differentiation. Although the (*pip*) × *pip* embryos die as young larvae and the presumptive ectoderm has been shown to have reduced competence, this does not necessarily indicate that such tissue is incapable of good differentiation when transplanted to a normal diploid host. Dalton (1946) has shown that transplanted haploid neural crest cells of *Triturus rivularis* can produce the normal pigment pattern, whereas the whole merogone reaches only the tailbud stage. Hadorn (1932, 1937) has shown that haploid *Triton palmatus* tissue is capable of normal, though retarded, differentiation, in contrast to the whole merogones, which die between tailbud and forelimb stage.

The diploid hybrid, *pip* × *syl*, showed reduced competence of the presumptive ectoderm when compared with the diploid and haploid *pipiens* embryos. Histological differentiation of *pip* × *syl* tissue is poor, even when incorporated with the host neural tissue (Moore, 1947). This is in marked contrast to transplants of lethal diploid tissue described by Lüthi (1938). He found that androgenetic *Triton palmatus* ♀ × *Salamandra maculosa* ♂ gastrula tissue would develop normally when transplanted into a diploid *T. palmatus* gastrula. The whole hybrid embryo develops no further than middle gastrula stage (Schönmann, 1938).

The complete lack of differentiation of the haploid hybrid tissue, (*pip*) × *syl*, is also in marked contrast to studies made by other workers. Haploid hybrid neural crest cells of *Triturus rivularis* ♀ × *Triturus torosus* ♂ have been transplanted by Dalton (1946) and were found capable of producing a pigment pattern, whereas the whole merogone developed only to the tailbud stage. De Roche (1937) found that transplanted presumptive ectoderm of androgenetic hybrid tissue between *Triton alpestris* ♀ × *Triton palmatus* ♂ developed normally, whereas whole merogones only developed as far as closed neural folds. Since these haploid hybrids differentiate at least as far as neurulae, their developmental capabilities should be greater than (*pip*) × *syl* tissue, which does not develop to a gastrula stage. Moreover, the diploid hybrids of these species reach metamorphosis, in contrast to the *pip* × *syl*, which die as early gastrulae.

Hadorn (1932) found that implanted androgenetic hybrid gastrula tissue of *Triton palmatus* ♀ × *Triton cristatus* ♂ formed normal tissue, except for pycnotic head mesenchyme. The whole merogones reached closed neural folds, with pycnotic head mesenchyme. In later experiments in which chimeras of androgenetic *T. palmatus* ♀ × *T. cristatus* ♂ gastrulae and diploid hybrid *T. palmatus* ♀ × *T. cristatus* ♂ gastrulae were studied, Hadorn (1937) found that the androgenetic hybrid tissue was incapable of differentiating anterior head structures. Additional experiments with the androgenetic hybrid material indicated that the ectoderm is incapable of forming an optic vesicle and that there may be reduced inductive ability of the head organizer. These latter experiments, which show a localized lack of competence, affecting anterior head structures, of (*palmatus*) × *cristatus* tissue, are of particular interest when compared with our experiments, which show no competence of (*pip*) × *syl* tissue. We would expect the developmental capabilities of (*palmatus*) × *cristatus* tissue to be considerably greater than that of (*pip*) × *syl* tissue, since diploid hybrids of *palmatus* × *cristatus* may metamorphose and the haploid hybrids reach closed neural folds.

From this comparison of similar transplantation experiments of other workers with ours, it is evident that the degree of differentiation of such transplants varies with the species used. When a comparison of transplants of diploid, haploid, diploid hybrid and haploid hybrid tissues, such as were described in this paper, is made, however, it is found that there is a gradation in the differentiating capabilities of such tissues, from a maximum for the diploid to none for the haploid hybrid tissue.

## SUMMARY

1. In an effort to dissociate the factors responsible for the failure of development in *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids, a study has been made of the development of haploid embryos composed of *pipiens* cytoplasm and *sylvatica* chromosomes. These androgenetic hybrids develop only to the late blastula stage.

2. The competence of the presumptive ectoderm of these haploid hybrids was tested by transplantation to neurulae of *Rana palustris*. The results indicated a total lack of competence.

3. Aside from the main problem with which the paper is concerned, it was found that the various classes of embryos used could be arranged in order of decreasing competence of the presumptive ectoderm. The sequence was as follows: diploid *pipiens*, haploid *pipiens*, *pipiens* ♀ × *sylvatica* ♂ diploid hybrids and (*pipiens* ♀) × *sylvatica* ♂ haploid hybrids.

## LITERATURE CITED

BALTZER, F., 1922. Über die experimentelle Erzeugung und Aufzucht eines haploiden *Triton taeniatus*. *Verh. Schweiz. Natf. Ges.,* **103**: 248–249.

DALTON, H. C., 1946. The role of nucleus and cytoplasm in development of pigment patterns in Triturus. *J. Exp. Zool.,* **103**: 169–200.

DE ROCHE, V., 1937. Differenzierung von Gewebe und ganzen Organen in Transplantaten der bastardmerogonischen Kombination *Triton alpestris* (♀) × *Triton palmatus* ♂. *Arch. f. Entw.,* **135**: 620–663.

FANKHAUSER, G., 1938. The microscopical anatomy of metamorphosis in a haploid salamander, *Triton taeniatus* Laur. *J. Morph.,* **62**: 393–413.

HADORN, E., 1932. Über Organentwicklung und histologische Differenzierung in transplantierten merogonischen Bastardgeweben (*Triton palmatus* (♀) × *Triton cristatus* ♂). *Arch. f. Entw.,* **125**: 495–565.

HADORN, E., 1937. Die entwicklungsphysiologische Auswirkung der disharmonischen Kern-Plasma-Kombination beim Bastardmerogon *Triton palmatus* (♀) × *Triton cristatus* ♂. *Arch. f. Entw.,* **136**: 400–489.

HOLTFRETER, J., 1933. Der Einfluss von Wirtsalter und verschiedenen Organbezirken auf die Differenzierung von angelagertem Gastrulaektoderm. *Arch. f. Entw.,* **127**: 619–775.

LÜTHI, H. R., 1938. Die Differenzierungsleistungen von Transplantaten der letalen Bastardkombination *Triton* ♀ × *Salamandra* ♂. *Arch. f. Entw.,* **138**: 423–450.

MOORE, A.-B. C., 1950. The development of reciprocal androgenetic frog hybrids. *Biol. Bull.,* **99**: 88–111.

MOORE, J. A., 1941. Developmental rate of hybrid frogs. *J. Exp. Zool.,* **86**: 405–422.

MOORE, J. A., 1946. Studies in the development of frog hybrids. I. Embryonic development in the cross *Rana pipiens* ♀ × *Rana sylvatica* ♂. *J. Exp. Zool.,* **101**: 173–220.

MOORE, J. A., 1947. Studies in the development of frog hybrids. II. Competence of the gastrula ectoderm of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. *J. Exp. Zool.,* **105**: 349–370.

MOORE, J. A., 1948. Studies in the development of frog hybrids. III. Inductive ability of the dorsal lip region of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. *J. Exp. Zool.,* **108**: 127–154.

PORTER, K. R., 1939. Androgenetic development of the egg of *Rana pipiens*. *Biol. Bull.,* **77**: 233–257.

SCHÖNMANN, W., 1938. Der diploide Bastard *Triton palmatus* ♀ × *Salamandra* ♂. *Arch. f. Entw.,* **138**: 345–375.

TING, H.-P., 1951. Diploid, androgenetic and gynogenetic haploid development in anuran hybridization. *J. Exp. Zool.,* **116**: 21–57.

# COMPOSITION OF THE SWIMBLADDER GAS IN DEEP SEA FISHES[1]

P. F. SCHOLANDER AND L. VAN DAM

*Woods Hole Oceanographic Institution, Woods Hole, Massachusetts,*
*and Lerner Marine Laboratory, Bimini, Bahamas*

In an earlier study of the composition of the swimbladder gas in bottom-dwelling marine fishes caught at depths down to 220 meters, it was found that at all depths the partial pressure of nitrogen in the swimbladder was usually close to that of the surrounding sea water, *i.e.,* 0.8 of an atmosphere (Scholander, Claff, Teng and Walters, 1951). $CO_2$ was present only in traces, and thus the pressure build-up above 0.8 atmosphere seemed to be due to oxygen only. A slight but definite increase in the nitrogen pressure with depth was apparent from the data, but this was considered at least partly to be an artifact due to changes in the gas composition after the catch.

Non-bottom-dwelling species were often found to have a partial pressure of nitrogen in their bladders far above 0.8 of an atmosphere. These fishes were in buoyancy equilibrium at a very shallow depth, and hence might have acquired their high nitrogen values when equilibrating at much lesser depths than that at which they were caught, *i.e.,* as a consequence of vertical migrations.

In the summer of 1952 we had the opportunity to extend the survey down to a depth of some 950 meters, *i.e.,* about four to five times deeper than before, with the result that now a fuller and more accurate picture of the performance of the gas gland in relation to depth can be presented.

## MATERIAL

Material for the present investigation was obtained at the Lerner Marine Laboratory, Bimini, Bahamas, December 24, 1951, to January 2, 1952, and during two trawling cruises from the Woods Hole Oceanographic Institution. The latter were conducted off the New England coast by the dragger *Cap'n Bill II* from July 10 to 17 and 23 to 30, 1952. Some 260 specimens were analyzed, covering 29 species of fish caught between 120 and 950 meters' depth.

At the Lerner Marine Laboratory the fishes were obtained at depths of 300–420 meters. They were caught at the bottom on a baited hook fastened to a heavily weighted wire line and were pulled to the surface within 3–5 minutes by means of a motor driven reel. The wire passed over a meter wheel which indicated the depth. Gas samples from the following species were analyzed:

| | |
|---|---|
| *Epinephelus* | Grouper |
| *Epinephelus mystacinus* | Black grouper |
| *Lutianus vivanus* | Long-fin red snapper |

---

[1] Contribution Number 632 from the Woods Hole Oceanographic Institution.

Fishing on the *Cap'n Bill* was done by means of an otter trawl, dragging the bottom for 30 minutes. The time of ascent of the net from the bottom to the surface may be estimated at about 30 minutes for the greatest depths. The depth of the stations was continuously indicated on a fathometer. Gas samples from the following fishes were taken and analyzed on board:

| | | Known depth range in meters |
|---|---|---|
| *Alepocephalus* sp. | | 890 |
| *Antimora viola* | Blue hake | 560–1830 |
| *Argentina silus* | Herring smelt | 60–670 |
| *Coryphaenoides rupestris* | Round-nose ratfish | 700–2200 |
| *Cottunculus thomsonii* | Deep sea sculpin | 190–1570 |
| *Dicrolene* sp. | | 840–890 |
| *Macrourus bairdii* | Common ratfish | 140–2300 |
| *Macrourus berglax* | Smooth-spined ratfish | 180–1240 |
| *Merluccius bilinearis* | Silver hake | 0–870 |
| *Notacanthus phasganorus* | | 870 |
| *Peristedion miniatum* | Deep sea robin | 170–350 |
| *Sebastes marinus* | Rosefish | 30–1680 |
| *Simenchelys parasiticus* | Slime eel | 360–1620 |
| *Synaphobranchus pinnatus* | Long-nosed eel | 240–2660 |
| *Tautogolabrus adspersus* | Cunner | 0–130 |
| *Urophycis chesteri* | Long-finned hake | 60–990 |
| *Urophycis chuss* | Squirrel hake (ling) | 0–540 |
| *Urophycis regius* | Spotted hake | 160 |
| *Urophycis tenuis* | White hake (ling) | 0–540 |

The data on the depth range are taken from Bigelow and Welsh (1924) and from Goode and Bean (1895), or are from our own records.

## Methods and Reliability of Data

The micro-gas-analytical method used gives the carbon dioxide and oxygen percentages through absorption with KOH and alkaline pyrogallol[2] respectively (Scholander *et al.*, 1951). The accuracy is about ± 0.2 to ± 0.3 per cent of the true value. The unabsorbed fraction ("nitrogen") of the gas sample, in addition to nitrogen, may contain noble gases and organic gases.

The amount of organic gases was determined by combustion in a separate chamber and subsequent analysis for $CO_2$ in the one-half cc. analyzer (Scholander, 1947). The plungers of the syringes with which the samples for these analyses were taken had been lubricated with concentrated lithium chloride solution, and care was taken throughout to avoid organic contaminations. The results are given in Table I and show that the amount of $CO_2$ formed by combustion of the swimbladder gas is usually no more than a few hundredths of one per cent. Hence organic gases play no significant role in the build-up of the gas pressure in the swimbladder of deep sea marine fishes. The traces of organic gases present in the samples may have been derived from bacterial activity in the gut of the fish.

---

[2] In the described technique the oxygen is absorbed by running a large excess of fresh pyrogallol down over the gas bubble. This procedure keeps carbon monoxide formation down to sub-detectable values, even in samples of pure oxygen, and agrees with results obtained by Kilday (1950), who used essentially the same absorption principles in a Haldane-type macromethod.

No carbon monoxide could be detected in gas samples from *Urophycis chesteri* and *Macrourus berglax.* Other species were not tested.

Schloesing and Richard (1896) analyzed the swimbladder gas of *Muraena helena* caught at 88 meters' depth and found that the argon over nitrogen-plus-argon ratio was 1.85%. Similarly in *Synaphobranchus pinnatus,* caught at 1385 meters' depth, the ratio was 1.94%. With a ratio in air of 1.18%, this means about a 60% increase in the argon over nitrogen-plus-argon ratio in these fishes. The samples were small and the analyses were not considered very accurate, although probably significant as to the increase mentioned. We have as yet no data on the content of argon in our material.

In our discussion of the correlation of the gas composition with depth we have assumed (a) that the fish are in a steady state of equilibrium at the depth of the catch, *i.e.,* that they have remained at this depth for a substantial period of time, and (b) that this gas composition has not changed materially since the time the fish was caught.

TABLE I

*Carbon dioxide content of swimbladder gas before and after combustion*

| Species | Depth of catch (m.) | % $N_2$ | % $CO_2$ | | $\Delta CO_2$ |
|---|---|---|---|---|---|
| | | | Before | After | |
| | | | Combustion | | |
| | | | (a) | (b) | (b)-(a) |
| *Antimora viola* | 820 | 7.8 | 1.31 | 1.32 | 0.01 |
| | 820 | 7.2 | 0.84 | 0.90 | 0.06 |
| *Coryphaenoides rupestris* | 720 | 10.0 | 0.18 | 0.19 | 0.01 |
| | 720 | 11.6 | 0.67 | 0.72 | 0.05 |
| | 720 | — | 0.85 | 0.96 | 0.11 |
| *Macrourus bairdii* | 820 | 8.2 | 0.03 | 0.16 | 0.13 |
| | 610 | 8.2 | 0.65 | 0.67 | 0.02 |
| *Sebastes marinus* | 440 | 11.2 | 0.37 | 0.38 | 0.01 |
| | 440 | — | 0.34 | 0.41 | 0.07 |
| *Synaphobranchus pinnatus* | 820 | 6.2 | 1.96 | 1.98 | 0.02 |
| *Urophycis tenuis* | 610 | 7.8 | 0.37 | 0.40 | 0.03 |
| | 610 | 5.8 | 0.20 | 0.20 | 0.00 |
| | 610 | 6.2 | 0.18 | 0.19 | 0.01 |

As to the first assumption, we know that the Bimini fishes were at the bottom when they were caught, and Bigelow and Welsh (1924) consider most of our northern deep sea species as decidedly bottom-dwelling species. To what extent these fishes might migrate up and down along the bottom slope is not known.

As to the second assumption, there is good evidence that the changes in gas composition that take place from the time the fish is caught until the time the gas sample is secured are so small that they could not change the conclusions significantly. Thus, the Bimini fishes, which were secured between five and ten times faster than the northern fishes, gave results which superimposed on the northern data. Two sets of experiments showed that changes in the gas composition after the catch occur only slowly. Bloated and more or less moribund individuals of eight species of our deep sea fishes, left on deck, in or out of water, showed an increase of at most one per cent in the nitrogen percentage in 30 minutes. In two species of fishes from much shallower depths, as reported earlier, the increase was larger, but still insignificant when compared with the total nitrogen.

In other experiments oxygen was injected into the peritoneal cavity of two remoras (*Echeneis naucrates*) and one puffer (*Spheroides maculatus*), caught in shallow water. The amount injected was about ten times the amount of swimbladder gas normally present in fishes of similar weights. In these fully alive fish the nitrogen per cent of the injected gas increased in half an hour by 1.4 and 0.4%, respectively. A fish at 1000 meters' depth contains 100 times as much gas as a surface fish, and if such an amount could have been injected into these remoras or the puffer the percentage nitrogen increase would have been entirely negligible.

Also, simple calculation shows that even if the entire amount of nitrogen dissolved in the tissues of a fish were transferred into the swimbladder, which was then expanded 100 times, the increase in the nitrogen would amount to only a few tenths of a per cent. Finally, the amount of oxygen present in a swimbladder at 100 atmospheres' depth is so great that respiratory oxygen removal could not materially change the nitrogen percentage either. We feel, therefore, that our data must reflect essentially the conditions which existed in the swimbladder when the fish was caught.

## COMPOSITION OF THE SWIMBLADDER GAS IN RELATION TO DEPTH

In Figure 1 a composite diagram is presented of the previous (Scholander *et al.*, 1951) and present data, extending from the surface forms to the deep sea forms at 950 meters. The diagonal straight line represents a nitrogen percentage which corresponds to a partial pressure of nitrogen of 0.8 atmosphere, *i.e.*, that of the surrounding sea water. We assume here that nitrogen values to the right of this line are due to something other than diffusion equilibrium and that the balance of the pressure is due to oxygen secretion. It is clear from the figure that the nitrogen values found at greater depths deviate increasingly from the line showing the atmospheric nitrogen tension.

Figure 2 shows the partial pressures of the nitrogen and oxygen as calculated from the percentages found and from the depths at which the fish were taken. In fishes living near the surface the nitrogen and oxygen tensions were generally found to be close to those in air, indicating that here the diffusion exchange between the swimbladder and the outside water dominates the picture. From the surface value of 0.8 atmosphere the nitrogen pressure increases in an over-all linear fashion with depth, often attaining values as much as 10 to 15 atmospheres higher than the nitrogen tension in the surrounding sea water.

Much of the considerable spread in the data in Figure 2 is due to specific differences among the fishes. In Figure 3 we have plotted the nitrogen for four species of fish, covering a wide range of depth. It is indicated that the increase in

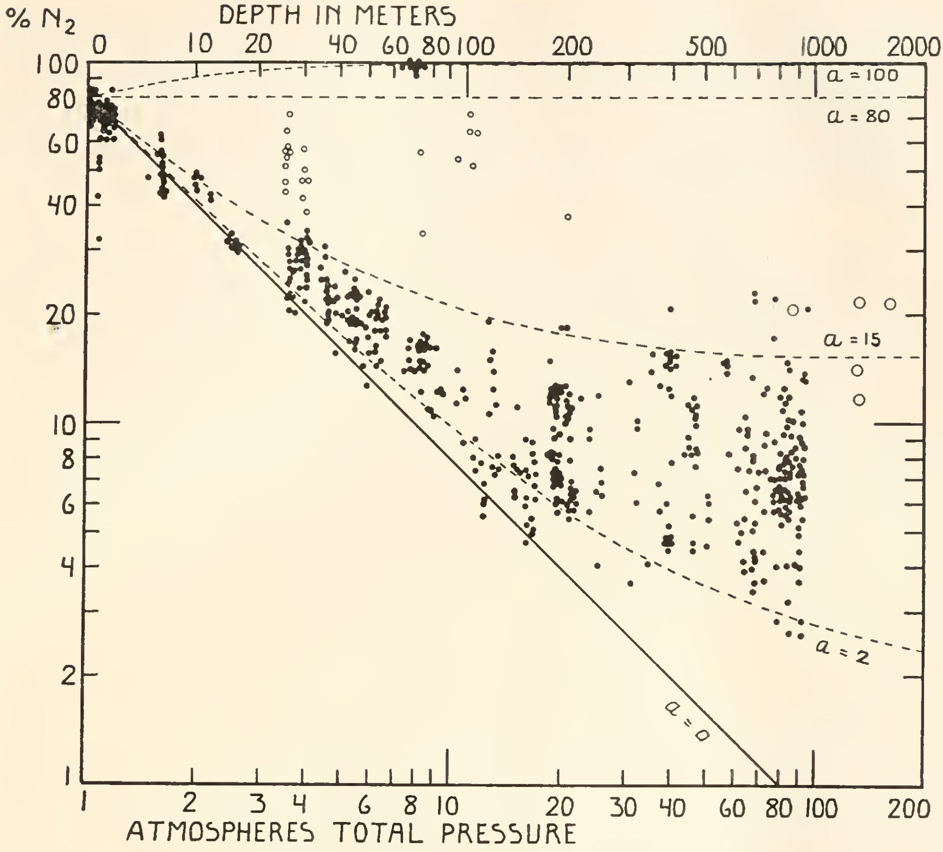


FIGURE 1. Composite graph of the nitrogen concentration found in swimbladder gas from fishes at various depths. The diagonal full-drawn line represents the percentage of nitrogen which at any given depth would give a partial pressure of nitrogen of 0.8 atmosphere. Both the present and previous data (Scholander *et al.*, 1951), as well as Hüfner's data (1892) for *Coregonus* (near 100% $N_2$), are given by solid circles. The large open circles are data on *Simenchelys parasiticus* (1674 m.) and *Synaphobranchus pinnatus*, given by Schloesing and Richard (1896). The small open circles are data from scup and silver hake, where the high nitrogen most likely is due to vertical migration. The dotted lines represent percentages of the total gas pressure due to nitrogen corresponding to values for $a$ of equation (1).. The empirical data indicate that $a$ in our material lies usually between 2 and 15.

nitrogen tension with depth in each species is more or less linear, the curves for the different species having individual slopes. These differences are also apparent in Figures 4 and 5.

*Sebastes* is a fish that apparently stays at the bottom in the daytime, but moves off the bottom at night. This could tend to produce a nitrogen value higher than that corresponding to the bottom depth (Scholander *et al.*, 1951). However, high nitrogen is also found in species like the deep sea sculpin, *Cottunculus,* and many others which are most likely always in close proximity to or on the bottom. Many of these fishes caught at depths of 900–1000 meters have a nitrogen percentage so high that they would have to move up to 100 meters' depth, or shallower, if their nitrogen were to be explained by simple diffusion from the sea water. Such vertical migrations are clearly out of the question for most of our fishes, and we are therefore forced to assume that nitrogen as well as oxygen can be deposited in the swimbladder against very considerable pressure gradients. This, of course, corroborates Hüfner's findings (1892) of nitrogen secretion in the whitefish.

## Relation Between Gas Deposition by Diffusion and Secretion

The swimbladder gas may be resolved into two main components. One is derived from diffusion exchange with the outside sea water. The other constitutes

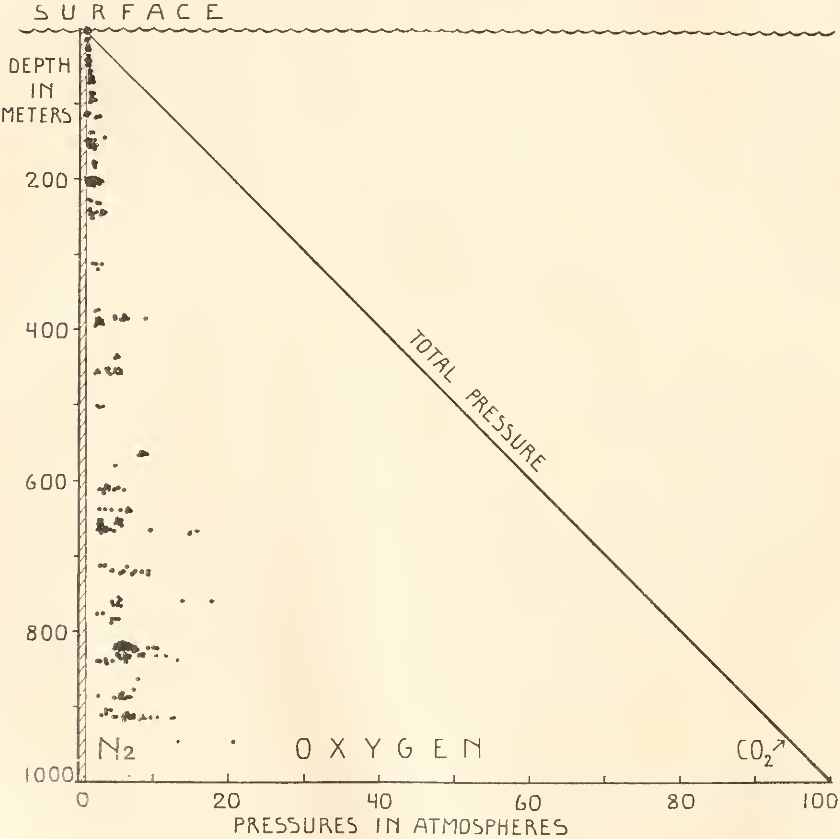


Figure 2. The partial pressures of nitrogen, oxygen and $CO_2$ calculated from the percentages and the depths. The shaded area is equal to the partial pressure of nitrogen in the sea water. The partial pressure of nitrogen in the swimbladder is to the left of each point, of oxygen to the right. The partial pressure of the $CO_2$ is usually less than what is represented by the thickness of the drawn diagonal line. The over-all picture suggests a linear increase in the partial pressure of the nitrogen with depth.

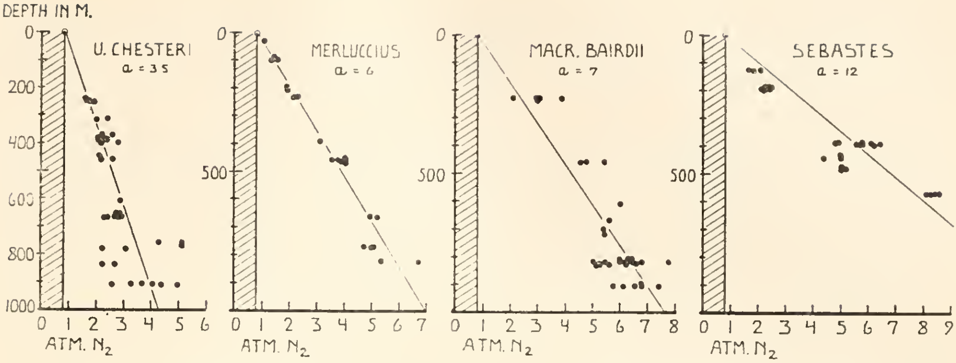


FIGURE 3. Partial pressures of nitrogen in the swimbladders of *Urophycis chesteri, Merluccius bilinearis, Macrourus bairdii,* and *Sebastes marinus.* The data suggest that the nitrogen in excess of 0.8 atmosphere increases linearly with depth from a value of zero at the surface. The different slopes of the lines indicate that the proportions of nitrogen and oxygen secreted are different in different species.

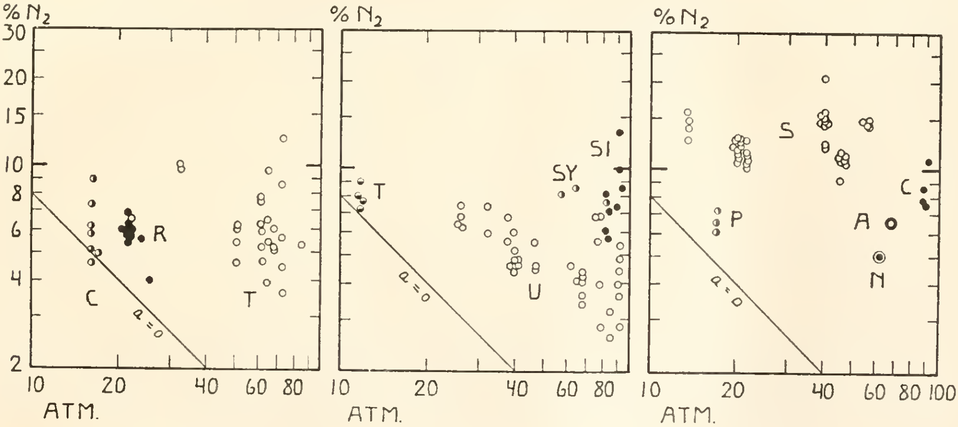


FIGURE 4. Gas composition in the swimbladder in relation to total pressure. Left: *Urophycis chuss* (C), *Urophycis regius* (R), *Urophycis tenuis* (T). Middle: *Tautogolabrus adspersus* (T), *Urophycis chesteri* (U), *Simenchelys parasiticus* (SI), *Synaphobranchus pinnatus* (SY). Right: *Peristedion miniatum* (P), *Sebastes marinus* (S), *Cottunculus thompsonii* (C), *Notacanthus phasganorus* (N), *Argentina silus* (A). These fishes were taken off the New England coast. The $N_2$ percentages of *Simenchelys* and *Synaphobranchus* obtained by Schloesing and Richard (1896) are entered on Figure 1. They are about twice as great as those shown from our data on these species.

what may be called the secreted [3] part which is responsible for the pressure build-up. As the nitrogen tension is known to be constant and very near to 0.8 atmosphere at all depths (Rakestraw and Emmel, 1938; Hamm and Thompson, 1941), we shall discuss below mainly the nitrogen deposition in the swimbladder. The same considerations may be applied also to the oxygen deposition, although here the picture may be somewhat blurred by the fact that the oxygen tension in the sea water varies considerably.

---

[3] We shall use the word "secretion" to mean any process whereby gases are deposited into the swimbladder so as to increase potentially or really the partial pressure above that in the ambient medium.

If a fish remains for a long time at a definite hydrostatic pressure where it maintains a neutral buoyancy, one may assume that the gas entering the swimbladder will be equal to that leaving the swimbladder in amount as well as in composition. Where such a steady-state situation obtains, the quantitative relation of the gas composition to the depth may be empirically described as the resultant of (1) a diffusion exchange term and (2) a gas secretion term.

The diffusion exchange component would lead to a partial pressure of nitrogen equal to the partial pressure in sea water, 0.8 atmosphere, times a factor, $F$, expressing the completeness of equilibrium between the swimbladder gas and the sea water, *i.e.*, $0.8 \times F$.

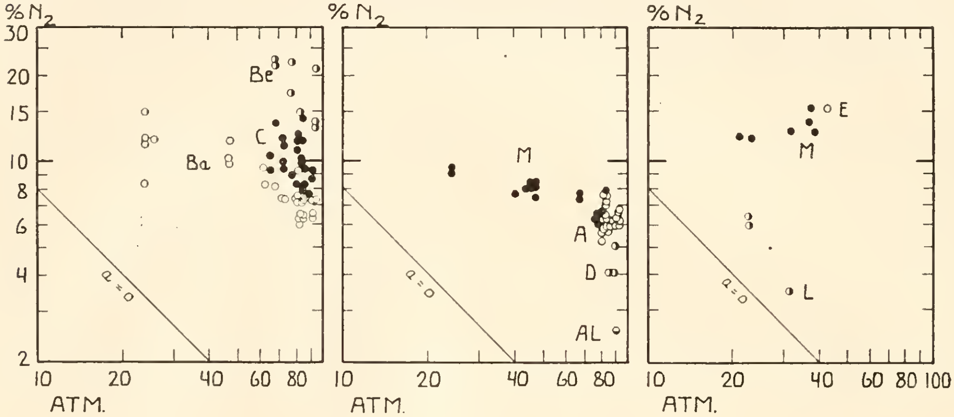


FIGURE 5. Gas composition in the swimbladder in relation to total pressure. Left: *Macrourus bairdii* (BA), *Macrourus berglax* (BE), *Coryphaenoides rupestris* (C). Middle: *Merluccius bilinearis* (M), *Antimora viola* (A), *Dicrolene* sp. (D), *Alepocephalus* (AL). Right: *Epinephelus mystacinus* (M), *Lutianus vivanus* (L), *Epinephelus* (E). The last three fishes were taken off Bimini. All the others were caught off the New England coast.

The gas secretion component represents an additional quantity of nitrogen which has entered the swimbladder as the result of secretion and which increases the partial pressure by an amount represented by $\frac{a}{100} \times D$, where $a$ is the percentage of nitrogen present in the swimbladder as the result of secretion and $D$ is the depth in atmospheres.

The total partial pressure, $P_{N_2}$, is thus given by

$$P_{N_2} = (0.8 \times F) + \left(\frac{a}{100} \times D\right) \qquad (1)$$

The factor $F$ is introduced into the diffusion term for the following reason. Near the surface $D$ is small and in most fishes the gas diffusion exchange with the sea water is so complete that a near-air composition is maintained in the swimbladder. This does not hold for all species, however. Even when kept in a shallow tank for a long time tautogs usually have much less than 80% $N_2$ in their swimbladders (Safford, 1940; Scholander *et al.*, 1951, Fig. 7). This fish, then, seems to have an exceptionally well developed isolation of its swimbladder with respect to diffusion, and this may be expressed by a factor $F$ which is smaller than one. Although difficult to verify, it seems likely that deep sea fishes would have developed a high degree of isolation of their swimbladders against diffusion (low $F$) as a means for reducing gas loss.

We may now scrutinize somewhat further the idea that the nitrogen deposited into the swimbladder to develop a pressure $\left(\frac{a}{100} D\right)$ is a more or less constant fraction of the oxygen pressure $\left(\frac{100 - a}{100} D\right)$. In Figure 1 we have plotted curves for a series of $a$ values (0, 2, 15, 80 and 100), according to equation (1). It will be seen that curves calculated using the $a$ values of 2 and 15 describe fairly well the upper and lower limits of nitrogen admixture in the deposited swimbladder gas of our marine fishes. A fish like *Coregonus* which deposits near to 100% nitrogen would be expected to present a nitrogen percentage increasing with depth. A fish with an $a$ value of 80 would maintain this percentage at all depths. We have no explanation why there seems to be a gap in the $a$ values between some 20 and 90–100. As indicated by Figure 3 and by Hüfner's data, $a$ seems to be a species characteristic.

Hüfner (1892) found that six out of nine whitefish (*Coregonus acronius*) from 60 to 80 meters' depth in the Bodensee had a nitrogen content in their swimbladders of 99–100%. At the surface these fishes were bloated with enough gas to make them buoyant at the bottom, and they must hence have deposited their nitrogen against a hydrostatic pressure of some 6–8 atmospheres (*cf.*, Scholander *et al.*, 1951). The data have been entered in Figure 1 and agree with the ideas embodied in equation (1). It is a pleasure to record that these seemingly incredible findings have lately been fully corroborated and extended by observations on fishes from the Great Lakes made by Mr. R. L. Saunders from the laboratory of Dr. F. E. J. Fry, Department of Zoology, University of Toronto.

## COMPOSITION OF THE GAS SECRETED

We have so far discussed the gas composition as it is found in the swimbladder at various depths, which leads us to believe that deep sea fishes deposit in their swimbladders not pure oxygen, but oxygen together with a fraction, $a$, of inert gases ("nitrogen"), $a$ being high in some species and low in others. We shall now consider to what extent the $a$ values might reveal what is the composition of the gas that has actually been secreted.

The gas pressure in the swimbladder in excess of the one atmosphere due to the combined pressure of nitrogen + oxygen in the sea water is the resultant of (1) the gas secretion into the bladder and (2) the diffusion losses through its wall. The swimbladder in deep sea fishes is capable of maintaining with low gas loss a pressure gradient of one hundred or more atmospheres through the thin air bladder wall, and it is therefore clear that the exit mechanism for the gases constitutes a potent diffusion barrier. It seems likely that the secreted gases at sub-maximal depths are produced with a pressure substantially higher than the ambient hydrostatic pressure. Only if the diffusion coefficients for the different gases were the same could the gas composition in the swimbladder equal the composition of the secreted gas. The diffusion coefficient for nitrogen through a variety of animal tissues has been found to be only around one half of that of oxygen (Krogh, 1919). The steady state requires that the molar amount of each gas leaving the bladder through diffusion loss should equal the molar amount secreted. A steady state can therefore occur only when the swimbladder has backed up enough partial pressure for each gas component to allow it to pass the diffusion barrier in an amount equal to that secreted. The pressures of nitrogen and oxygen accumulated in the swimbladder above the ambient partial pressures were empirically found to be $\frac{a}{100} \times D$ atmospheres and $\frac{100 - a}{100} \times D$ atmospheres respectively (equation (1)). The diffusion losses, and therefore secretion, of these gases would hence be $\frac{a}{100} \times D \times n$ and $\frac{100 - a}{100} \times D \times o$, where $n$ and $o$ are the diffusion coefficients for nitrogen and oxygen respectively. Recalculated on a per cent basis, the secreted nitrogen percentage, $N_{2_s}$, would relate to $a$ according to

$$N_{2_s} = \frac{100\,a}{\frac{o}{n}(100 - a) + a}. \tag{2}$$

With the ratio of $o/n = 2/1$, the secreted $N_2$ percentage would be $\frac{100\,a}{200 - a}$. This means that for low values of $a$, like the ones found, it might be anticipated that the secreted mixture would have a *nitrogen* percentage equal to about $\frac{1}{2}\,a$. Conversely, if $a$ were near 100, as found in *Coregonus,* the *oxygen* percentage of the secreted gas would be about twice as high as the oxygen percentage found. Thus, if the nitrogen percentage in the swimbladder of a fish at 1000 meters' depth is found to be 4.8%, then $a$ is 4.0% and the secreted mixture is likely to be 2% $N_2$ and 98% $O_2$. If in a *Coregonus* the $a$ value is 95, the secreted gas is likely to be 90% $N_2$ and 10% oxygen.

Schloesing and Richard (1896) found the argon fraction in the swimbladder "nitrogen" to be some 60% higher than in air. From the solubility of argon in water and its molecular weight one would expect argon to have twice as high a diffusion coefficient through animal tissue as nitrogen. If, therefore, argon and nitrogen entered the swimbladder in the same ratio as in air one would expect a lower argon fraction in the swimbladder rather than a higher. Evidently, therefore, these two gases enter the swimbladder in proportions different from those in air. We are totally in the dark as to how the presumably inert argon and nitrogen have attained the high pressures found in the swimbladder.

We wish to express our gratitude to Dr. Alfred C. Redfield for his stimulating interest. We were fortunate to have all possible cooperation from Dr. William C. Schroeder, who conducted the deep sea cruises off the New England coast. He identified our specimens and supplied us with information on the depth ranges of the fishes. The Captain and the crew of the dragger *Cap'n Bill II* did everything to facilitate our work on board.

We are indebted to Dr. C. M. Breder, Jr., at the American Museum of Natural History, for arranging our stay at the Lerner Marine Laboratory. We wish to extend our thanks to Mr. Michael Lerner for his generous cooperation in securing deep sea material at Bimini and to Mr. Marshall Bishop for providing us with the most excellent facilities in the laboratory. We wish to thank Mr. Vladimir Walters, of New York University, for his excellent assistance, and we are much obliged to Mrs. Susan I. Scholander for help in the field and in the preparation of the final manuscript.

## SUMMARY

1. The composition of the swimbladder gas has been determined in 26 species (260 specimens) of marine deep-sea fishes taken at known depths between 200 and 950 meters.

2. The partial pressure of nitrogen in the bladder steadily increases with depth until it reaches some 5–15 atmospheres at a depth of 900 meters, revealing that not only oxygen but also nitrogen is transported into the swimbladder against a considerable gradient. This corroborates the findings of Hüfner (1892) that the whitefish (*Coregonus*) is able to secrete pure nitrogen against a hydrostatic pressure of 6–8 atmospheres.

3. The data, together with previous observations from shallower depths, show that the nitrogen tension at all depths may be expressed by (1) a diffusion term of 0.8 atmosphere, plus (2) a secretion term which, although different for different species of fish, is in each species a constant percentage of the total secreted gas pressure. Among our fish the secretion term ranges from about 2% to 15% nitrogen. In the whitefish it is apparently 100%. In a steady-state situation the percentage nitrogen actually secreted into the swimbladder is probably lower than the nitrogen percentage indicated by the secretion term because oxygen is more easily lost by diffusion through animal tissue than is nitrogen.

## LITERATURE CITED

Bigelow, H. B., and W. W. Welsh, 1924. Fishes of the Gulf of Maine. Bull. U. S. Bureau Fisheries, vol. 40, pt. 1.

Goode, G. B., and T. H. Bean, 1895. Oceanic ichthyology. Smithsonian Contr. Knowledge, vols. 30–31.

Hamm, R. E., and T. G. Thompson, 1941. Dissolved nitrogen in the sea water of the Northeast Pacific with notes on the total carbon dioxide and the dissolved oxygen. *J. Marine Res.*, **4**: 11–27.

Hüfner, G., 1892. Zur physikalischen Chemie der Schwimmblasengase. *Arch. Anat. Physiol., Physiol. Abth.*, 54–79.

Kilday, Marthada V., 1950. A quantitative study of the carbon monoxide formed during the absorption of oxygen by alkaline pyrogallol. *J. Research Natl. Bureau Standards*, **45**: 43–59.

Krogh, A., 1919. The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.*, 52: 391–408.

RAKESTRAW, N. W., AND V. M. EMMEL, 1938. The relation of dissolved oxygen to nitrogen in some Atlantic waters. *J. Marine Res.*, **1**: 207–216.

SAFFORD, VIRGINIA, 1940. Asphyxiation of marine fish with and without $CO_2$ and its effect on the gas content of the swimbladder. *J. Cell. Comp. Physiol.*, **16**: 165–173.

SCHLOESING, T., AND J. RICHARD, 1896. Recherche de l'argon dans les gaz de la vessie natatoire des poissons et des physalies. *C. R. Acad. Sci. Paris*, **122**: 615–617.

SCHOLANDER, P. F., 1947. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. Biol. Chem.*, **167**: 235–250.

SCHOLANDER, P. F., C. L. CLAFF, C. T. TENG AND V. WALTERS, 1951. Nitrogen tension in the swimbladder of marine fishes in relation to the depth. *Biol. Bull.*, **101**: 178–193.

# BODY TEMPERATURES OF WHITE-FOOTED MICE IN RELATION TO ENVIRONMENTAL TEMPERATURE AND HEAT AND COLD STRESS [1]

JOHN A. SEALANDER, JR.

*Department of Zoology, University of Arkansas, Fayetteville, Arkansas*

Many mammals are completely homoiothermic only with a relatively narrow range of external temperature, particularly some of the more primitive mammals which, with respect to thermoregulatory ability, are intermediate between the true poikilotherms and the more highly evolved mammals (Britton and Atkinson, 1938; Kredl, 1928; Martin, 1930; Morrison, 1945, 1946; Sutherland, 1897; Wislocki and Enders, 1935). Body temperatures of more advanced mammals are influenced by changes in air temperature but do not undergo the wide fluctuations that are characteristic of primitive mammals (Gaalaas, 1945; Seath and Miller, 1946). Among mammals the bats seem to be distinct in that, except during periods of activity, they appear to be essentially poikilothermic (Hock, 1951; Reeder and Cowles, 1951).

As a group the rodents show varying degrees of perfection of thermoregulation. The most labile temperatures are generally found among the hibernating species, although some non-hibernators also have inconstant body temperatures (Morrison and Ryser, 1951; Wade, 1930; Wislocki, 1933). The term "heterotherm" has been applied to certain rodents and other mammals which show rather wide fluctuations in body temperatures in relation to the ambient temperature as contrasted with the more perfectly regulating homoiotherms.

Various investigators have attempted to relate body temperatures of rats and mice to air temperatures and have pointed out differences due to both air temperature and sex (Bierens de Haan, 1922; Hart, 1951a, 1951b; Herrington, 1940; Morrison and Ryser, 1951; Przibam, 1917; Sumner, 1913).

Early studies on the effect of previous acclimation at high and low temperatures on the thermoregulatory ability of rodents and other mammals are few (Gelineo, 1934, 1940; Schwabe, Emery and Griffith, 1938). Only within recent years has this problem been studied intensively (Adolph, 1950, 1951; Adolph and Lawrow, 1951; Scholander *et al.,* 1950; and others). However, many of the mechanisms involved in acclimation to heat and cold still remain obscure.

## METHODS AND MATERIALS

The mice used in this study were northern white-footed mice, *Peromyscus leucopus noveboracensis* (Fischer), which were trapped from wild populations in the vicinity of Champaign-Urbana, Illinois, during 1947 to 1949. All individuals were acclimated in the laboratory at temperatures from 20–24° C. for periods of 20–30 days before any body temperature measurements were made.

---

[1] Contribution from the Zoological Laboratory, University of Illinois, Urbana. The author wishes to express appreciation to Dr. S. C. Kendeigh for his sympathetic guidance in carrying out this study.

The temperature chambers used in these experiments have been described in a previous paper (Sealander, 1951a).

Deep body temperatures were measured with a portable indicator potentiometer using fine copper-contantan thermocouples. The usual practice was to insert the thermocouple into the animal's esophagus, since it was presumed that possible damage to internal tissues might be less and because less difficulty was experienced in securing temperatures in this way. Thermocouples were periodically checked for accuracy against a standard thermometer.

As activity exerts an appreciable influence on body temperature (Britton and Kline, 1939; Bullough, 1949; Hart, 1951a; Kendeigh, 1945), a sufficient number of records was usually obtained to secure both maximum and minimum temperatures. A series of temperatures was taken at about one-minute intervals until no further changes in temperature could be detected for several minutes. Temperatures were recorded while the animal was held in the left hand or confined in a small cone of wire netting. The animals were handled with a glove to minimize any changes in body temperature due to radiation or conduction of heat from the handler. The mice struggled briefly when the thermocouple was inserted and then became quiet. If they continued to struggle no additional readings were taken. Body temperatures were almost invariably highest during the first minute or two of recording and then underwent only minor fluctuations during the remaining period of measurement. After the initial rise, body temperatures continued to decline gradually and usually from 8–12 minutes elapsed before they remained constant. Body temperatures of mice subjected to low temperatures were recorded within about a ten-minute period to preclude any rise in temperature from exposure to warmer room temperatures. Body temperature readings followed the same general pattern in these mice.

Automatically recorded subcutaneous temperatures of mice at different air temperatures were obtained with thermocouples inserted under the skin (Sealander, 1951b) which were connected to a strip-chart potentiometer that recorded the balance point of the circuit every three minutes.

## Experimental Results

*Behavior responses.* Reactions of individual white-footed mice to temperature stress were, in general, quite similar to those which have been frequently reported for laboratory rats, mice, hamsters and guinea pigs.

Initial exposures at moderately low temperatures (– 10° to – 15° C.) were accompanied by periods of gnawing at the cage alternating with vigorous grooming movements. Motor activity was evidenced by frequent exploratory trips to different corners of the cage. Lowering the temperature to about – 20° C. intensified these behavior responses for a short while, but gnawing and motor activity soon became infrequent although grooming movements persisted. At air temperatures between – 20° and – 30° C. there were only occasional outbursts of motor activity in the form of running.

Erection of the fur and reduction of the amount of exposed surface by curling into a ball became more prominent as the mice became quiet. Frequent turning movements were made, apparently in an effort to achieve a more compact body surface. At very low temperatures (– 25° to – 35° C.) the feet, ears and tail quite often became frost-bitten due to greater exposure and contact with cold surfaces.

Polypnea and shivering were characteristic responses to cold. Their onset varied with individual mice and with the suddenness of exposure to low temperatures. Rapid breathing and shivering movements were most prominent at very low temperatures but appeared rather abruptly in mice that were suddenly placed at temperatures of about − 20° C. Respiration became weak and shallow and shivering disappeared in hypothermal mice. Loss of equilibrium appeared with the approach of the lower lethal body temperature, and the lethal point was often marked by a final weak burst of activity followed by a total loss of balance and culminated in a clonic-tonic convulsion of the type described by Frings and Frings (1952).

At temperatures of 25° C. and above the mice decreased their motor activity and generally assumed sleepy, relaxed postures. When air temperature was around 35° C. the extremities became noticeably suffused with blood. At 40° C. motor activity was usually quite pronounced during the first half-hour of exposure but then ceased almost entirely. The mice salivated quite freely at this temperature and often assumed a partially erect posture with the forepaws resting upon the sides of the cage. In the neighborhood of 50° C. crawling movements were evident and the belly fur became streaked and matted with saliva as the mice crawled over the bottom of the cage.

Death occurred very rapidly in the range of temperature between 40° and 50° C. but was not accompanied by the convulsive seizures and anoxic symptoms noted at low temperatures.

*Minimal recoverable body temperatures.* The extremes of low temperature which many species of mammals can endure for a short period of time and still survive are often very great. For example, the laboratory rat is able to maintain a constant body temperature for several hours at − 30° C. (Giaja and Gelineo, 1933). Many species of arctic mammals are notably resistant to cold as pointed out by Scholander and his associates (1950).

A number of experiments have been performed (Barbour *et al.,* 1943, 1944; Britton, 1922; Britton and Atkinson, 1938; Haterius and Maison, 1948; Luyet and Gehenio, 1940) in which mammals were subjected to very low temperatures and underwent recovery. In the majority of mammals body temperatures from about 14° to 20° C. were the lowest which could be tolerated for any length of time with complete spontaneous recovery at room temperatures, but bats and ground squirrels tolerated body temperatures from a few tenths to about two degrees below 0° C. Other mammals, such as hamsters (Adolph, 1951; Adolph and Lawrow, 1951), tolerate body temperatures as low as 4° C. for varying lengths of time.

Very few minimal recoverable body temperatures have been determined for different species of mice. Sumner (1913) exposed white mice to air temperatures of − 2° to 2° C. for about five hours and noted complete recovery of two mice from temperatures of 20.6° and 12.5° C. after exposure to an air temperature of 28.5° C. Kendeigh (1945) reported the recovery of a white-footed mouse, *Peromyscus maniculatus gracilis,* after its body temperature had fallen to 23.4° C. after several hours exposure in a trap.

In the present study no systematic attempt was made to determine minimal recoverable body temperatures but a few incidental observations were obtained.

Two male white-footed mice exposed to air temperatures from − 12° to − 23° C. had body temperatures of 10° and 23° C., with exposures of 65 and 80 minutes, respectively, when removed to room temperatures of 26–27° C. Both underwent complete spontaneous recovery. Another male exposed to − 20° C. for 70 minutes underwent partial recovery from a body temperature of 6° C. when removed to a room temperature of 27° C. but subsequently died. These few records indicate that this species probably falls within the general range of tolerance for non-hibernating rodents of the same general size class. There was little or no activity by the mice during the time they were exposed to the low temperature.

*Lethal temperatures.* Upper and lower air temperature limits between which mammals can remain completely homoiothermal over long periods of time are but incompletely known for many species. In general, these limits vary with the animal's total heat production and the degree of body insulation.

Table I

*Esophageal temperatures of nineteen adult Peromyscus leucopus noveboracensis at room temperatures of 25° to 28° C.*

| Females | | | | Males | | | |
|---|---|---|---|---|---|---|---|
| Number of records | Temperature in ° C. | | | Number of records | Temperature in ° C. | | |
| | Minimum | Maximum | Mean | | Minimum | Maximum | Mean |
| 1 | — | 38.7 | — | 10 | 35.8 | 39.2 | 37.0 |
| 8 | 35.5 | 39.0 | 38.3 | 1 | — | 38.2 | — |
| 1 | — | 39.5 | — | 9 | 37.0 | 40.5 | 38.2 |
| 9 | 37.5 | 39.6 | 37.9 | 11 | 36.0 | 38.7 | 37.3 |
| 8 | 37.3 | 39.8 | 38.5 | 9 | 35.5 | 39.3 | 37.4 |
| 10 | 36.8 | 40.5 | 38.3 | 8 | 38.7 | 39.5 | 38.9 |
| 10 | 37.2 | 39.7 | 38.1 | 10 | 36.0 | 39.0 | 37.5 |
| 12 | 36.3 | 38.7 | 37.7 | 12 | 35.7 | 37.8 | 37.0 |
| 110 | 35.7 | 40.2 | 38.2 | 12 | 36.1 | 37.6 | 37.3 |
| | | | | 108 | 35.4 | 38.3 | 37.7 |
| Mean | 36.6 | 39.5 | 38.1 | | 36.2 | 38.8 | 37.6 |

Rodents are known to be much more susceptible to high temperatures than many other mammals. The usual explanation for this greater susceptibility is their lack of sweat glands, coupled with a high respiratory rate that cannot be increased greatly for cooling.

A series of esophageal temperatures taken on 15 adult *Peromyscus leucopus,* of both sexes, at the moment of death as indicated by the last convulsive respiratory movement, gives some indication of the lower lethal body temperature for this species. The mice were subjected to temperatures ranging from − 12° to − 35° C. while deprived of food and water. Lower lethal body temperatures ranged from 3.5° to 5.0° C. with a mean of 4.6° ± .5° C. In general, the duration of exposure before death occurred showed a progressive increase (45 minutes to 8.5 hours) with rise in temperature. However, there was no evident correlation between the lower lethal body temperature and either air temperature or length of exposure. Inasmuch as body temperature changes rapidly after thermogenic ability is once lost it is difficult to fix an absolute lethal temperature. Heat production is probably permanently impaired in deer mice at body temperatures in the neighborhood of 10° C. as evidenced by lack of permanent recovery from temperatures in this vicinity.

A few experiments were performed in which deer mice were subjected to falling or gradually lowered air temperatures. In all cases the body temperature underwent the most rapid decline when the ambient temperature was around − 12° to − 15° C. All of the animals concerned in the experiments were freshly caught summer animals. Although it would appear that this is a critical zone of temperature for summer animals it seems probable that the critical zone might be lower for animals previously acclimated at low environmental temperatures.

TABLE II

*Comparison of esophageal and subcutaneous temperatures of seven adult Peromyscus leucopus noveboracensis at 25° C. when under anaesthesia* and when fully recovered***

| Sex | Body Temperature (° C.) | | | |
|---|---|---|---|---|
| | Under light anaesthesia at point of recovery | | Two hours after recovery from anaesthesia | |
| | Mean esophageal temperature | Mean subcutaneous temperature | Mean esophageal temperature | Mean subcutaneous temperature |
| F. | 37.1(3) | 35.9(3) | 38.4(4) | 37.2(4) |
| M. | 36.7(4) | 35.5(4) | 37.5(5) | 36.0(5) |
| F. | 36.5(5) | 35.3(5) | 37.6(5) | 35.9(5) |
| M. | 37.5(5) | 37.1(5) | 37.8(5) | 37.0(5) |
| M. | 36.7(5) | 35.0(5) | 38.2(4) | 36.9(4) |
| F. | 37.5(4) | 36.1(4) | 38.3(5) | 37.1(5) |
| M. | 36.9(4) | 35.5(4) | 37.0(5) | 36.1(5) |
| Mean | 37.0 | 35.8 | 37.8 | 36.6 |
| Mean difference | 1.2 | | 1.2 | |

* Sodium pentobarbital (Nembutal).

** Numbers in parentheses indicate number of records averaged. Readings taken at approximately half-minute intervals.

In experiments which involved a male and a female deer mouse, brief attention was given to the effect of high air temperatures on body temperature. These mice were exposed to air temperatures ranging from 40° to 50° C. for varying lengths of time. These experiments indicated that the upper limit of body temperature which could be tolerated for any length of time by *Peromyscus leucopus* was somewhere between 42° and 43° C. Apparently such high body temperatures can be tolerated for only brief periods without permanent impairment of temperature regulation. Exposure to temperatures of 45° and above resulted rather rapidly, usually in the space of about one-half hour, in impairment of thermoregulation. No return to normal body temperatures took place after exposure to air temperatures of 45° to 50° C. Non-reversible upper lethal temperatures between 43° and 44° were obtained for both of the experimental animals. These results are in general accord with those for the rat (Adolph and Lawrow, 1951) and for similar sized mice (Bodenheimer, 1949; Fuller and Hiestand, 1947).

*Normal body temperatures.* A series of esophageal temperatures was taken on several individual mice at non-stimulating air temperatures (Table I). The minimum or lowest reading obtained for each individual is considered to represent resting conditions, while the mean possibly indicates body temperatures of mice when in an active state and performing various routine activities in their natural environment. The mean body core temperature seems to lie between 37° and 38° C. The body temperature of all female mice averaged 0.5° C. higher than that of males, although in a few cases individual means were lower. The complete range of variability of body temperature in the mice measured was from 2.1° to 4.5° C. with a mean of 3.0° in the females, and from 0.8° to 3.8° C. with a mean of 2.6° in the males. Variation in body temperature became very much reduced when the mice were quiet and body temperature fluctuations were then in the neighborhood of ± 0.5° C.

TABLE III

*Esophageal temperatures of Peromyscus leucopus noveboracensis in relation to air temperature with and without food and water*

| Mean air temperature (° C.) | Sex and number | Hour of Exposure | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | | Mean esophageal temperature with food and water provided | | | | | | | | | | | |
| −30 | M 10b | 30.5 | 20.1 | 10.2 | | | | | | | | | |
| −30 | F 7c | 32.7 | 23.4 | 9.9 | | | | | | | | | |
| −20 | F 8c | 38.7 | 38.3 | 38.4 | 38.2 | 38.1 | 38.0 | 37.9 | 38.0 | 38.2 | 38.1 | 38.0 | 38.1 |
| −20 | F 9c | 38.0 | 38.1 | 38.2 | 38.4 | 38.5 | 38.2 | 38.0 | 38.1 | 37.9 | 37.7 | 37.8 | 37.9 |
| −14 | M 11b | 37.6 | 37.6 | 37.8 | 37.4 | 37.8 | 37.9 | 38.0 | 38.1 | 38.1 | 38.0 | 37.9 | 37.9 |
| −14 | F 10c | 38.0 | 38.1 | 38.1 | 38.2 | 37.9 | 38.1 | 37.8 | 37.9 | 37.8 | 38.0 | 37.9 | 38.0 |
| − 1 | M 12b | 37.5 | 37.6 | 37 9 | 38.0 | 37.9 | 37.7 | 38.1 | 37.9 | 37.9 | 37.7 | 37.8 | 37.9 |
| − 1 | M 13b | 38.1 | 38.2 | 38.0 | 38.4 | 38.3 | 38.5 | 38.2 | 38.1 | 38.0 | 37.9 | 38.0 | 38.1 |
| 25 | F 11c | 38.4 | 38.2 | 38.0 | 38.2 | 38.2 | 38.5 | 38.3 | 38.1 | 38.0 | 37.9 | 38.4 | 38.3 |
| 25 | M 14b | 37.9 | 37.6 | 37.7 | 37.4 | 37.7 | 37.8 | 37.7 | 37.6 | 37.4 | 37.8 | 37.9 | 38.0 |
| | | Mean esophageal temperature without food and water | | | | | | | | | | | |
| −30 | M 15b | 31.7 | 14.5 | | | | | | | | | | |
| −30 | F 12c | 36.3 | 16.8 | | | | | | | | | | |
| −20 | F 8c | 38.8 | 37.9 | 23.9 | | | | | | | | | |
| −20 | F 10c | 37.6 | 37.1 | 20.1 | | | | | | | | | |
| −12 | M 11b | 37.8 | 37.6 | 37.3 | 37.7 | 37.9 | 38.1 | 37.8 | 37.5 | 30.1 | 11.7 | | |
| −12 | F 9c | 38.1 | 38.0 | 38.5 | 38.1 | 37.9 | 38.3 | 38.3 | 32.2 | 14.6 | | | |
| 0 | M 12b | 37.8 | 37.9 | 37.6 | 37.3 | 37.1 | 37.0 | 38.0 | 37.7 | 37.5 | 37.5 | 37.4 | 37.4 |
| 0 | M 13b | 38.4 | 38.6 | 38.7 | 38.8 | 38.6 | 38.2 | 38.1 | 38.0 | 38.4 | 38.1 | 38.5 | 38.4 |
| 26 | F 11c | 37.9 | 38.1 | 38.3 | 38.0 | 38.0 | 38.5 | 38.0 | 37.9 | 37.8 | 38.1 | 38.1 | 38.2 |
| 25 | M 14b | 37.6 | 37.5 | 38.2 | 38.1 | 38.0 | 37.9 | 37.9 | 37.7 | 37.9 | 37.6 | 37.5 | 37.6 |

*Esophageal and subcutaneous temperature differences.* It is well known that gradients in temperature exist between the interior of the body and the surface or subcutaneous area, due to differences in rates of heat loss and heat production in these regions. The difference between subcutaneous and esophageal temperatures in white-footed mice was determined by alternately measuring esophageal and subcutaneous temperatures at approximately half-minute intervals with an indicator potentiometer. While at room temperature, temperatures were recorded from the same animals when under light anaesthesia and two hours after recovery from anaesthesia (Table II). Individual differences between esophageal and subcutaneous temperatures ranged from 0.4° to 1.7° for anaesthetized animals and from 0.9° to 1.7° for those which had recovered from anaesthesia. The mean difference between subcutaneous and esophageal of anaesthetized animals was the same as that of animals which had recovered from anaesthesia.

TABLE IV

*Subcutaneous temperatures of Peromyscus leucopus noveboracensis in relation to air temperature with and without food and water*

| Mean air temperature (° C.) | Sex and number | Hour of Exposure | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | | Mean subcutaneous temperature with food and water provided | | | | | | | | | | | |
| −30 | F 1c | 36.3 | 26.4 | 10.7 | | | | | | | | | |
| −30 | M 1b | 37.2 | 25.7 | 9.9 | | | | | | | | | |
| −30 | M 2b | 32.4 | 28.5 | 5.3 | | | | | | | | | |
| −25 | M 3b | 36.5 | 36.7 | 36.0 | 35.9 | 36.9 | 37.0 | 37.3 | 37.0 | 36.5 | 36.7 | 36.8 | 37.1 |
| −24 | F 2c | 37.5 | 38.7 | 39.1 | 38.7 | 38.3 | 37.9 | 37.4 | 37.4 | 37.6 | 36.9 | 36.3 | 35.8 |
| −24 | M 4b | 39.1 | 38.1 | 37.5 | 37.4 | 36.8 | 36.6 | 34.1 | 34.1 | 33.9 | 29.5 | 4.6 | |
| −22 | F 3c | 35.8 | 37.1 | 38.6 | 38.6 | 38.3 | 37.9 | 38.2 | 37.8 | 36.6 | 35.4 | 26.3 | 4.1 |
| −12 | F 3c | 38.4 | 38.2 | 38.0 | 37.9 | 38.2 | 38.6 | 38.5 | 38.6 | 38.1 | 38.0 | 38.1 | 38.2 |
| −12 | F 4c | 37.7 | 37.8 | 37.6 | 37.8 | 37.9 | 37.9 | 38.0 | 37.8 | 37.7 | 37.7 | 37.9 | 37.8 |
| 26 | M 1b | 37.4 | 37.2 | 37.1 | 37.6 | 37.5 | 37.5 | 37.4 | 37.2 | 37.0 | 37.1 | 37.2 | 37.2 |
| 26 | F 4c | 37.9 | 38.0 | 38.0 | 38.1 | 38.3 | 38.1 | 37.9 | 37.8 | 37.7 | 37.8 | 37.6 | 37.9 |
| | | Mean subcutaneous temperature without food and water | | | | | | | | | | | |
| −30 | F 5c | 30.8 | 11.5 | | | | | | | | | | |
| −30 | M 5b | 29.7 | 9.8 | | | | | | | | | | |
| −20 | F 6c | 38.2 | 34.7 | 10.5 | | | | | | | | | |
| −20 | M 6b | 37.3 | 28.6 | 10.2 | | | | | | | | | |
| −18 | M 7b | 37.3 | 33.5 | 13.9 | | | | | | | | | |
| −14 | M 8b | 33.9 | 33.5 | 33.9 | 33.5 | 36.6 | 34.3 | 11.9 | | | | | |
| −12 | M 9b | 37.2 | 36.4 | 36.7 | 35.7 | 35.7 | 38.3 | 37.9 | 30.9 | 12.7 | | | |
| − 1 | M 6b | 37.2 | 37.7 | 37.4 | 37.3 | 37.0 | 37.8 | 37.5 | 37.4 | 37.7 | 37.6 | 37.3 | 37.6 |
| − 1 | F 6c | 38.4 | 38.0 | 38.1 | 38.3 | 38.2 | 38.0 | 38.5 | 38.3 | 38.4 | 38.1 | 38.1 | 38.2 |
| 28 | M — | 37.6 | 37.7 | 36.1 | 37.0 | 36.8 | 36.9 | 36.3 | 37.0 | 36.5 | 36.2 | 37.3 | 39.3 |
| 27 | F — | 37.0 | 36.9 | 37.3 | 37.2 | 37.7 | 37.7 | 38.3 | 38.6 | 38.1 | 37.9 | 38.0 | 38.0 |

No simultaneous comparisons between esophageal and subcutaneous body temperatures were made at low air temperatures, but some idea of the relationship between the two temperatures in active, unanaesthetized animals can be obtained by comparing individual determinations of esophageal and subcutaneous temperatures at different air temperatures (Tables III and IV). However, as the same individuals were not used in the two series of determinations, temperature differences cannot be determined for individual mice.

*Body temperature in relation to cold stress.* Both esophageal and subcutaneous temperatures were determined at different degrees of low temperature and both

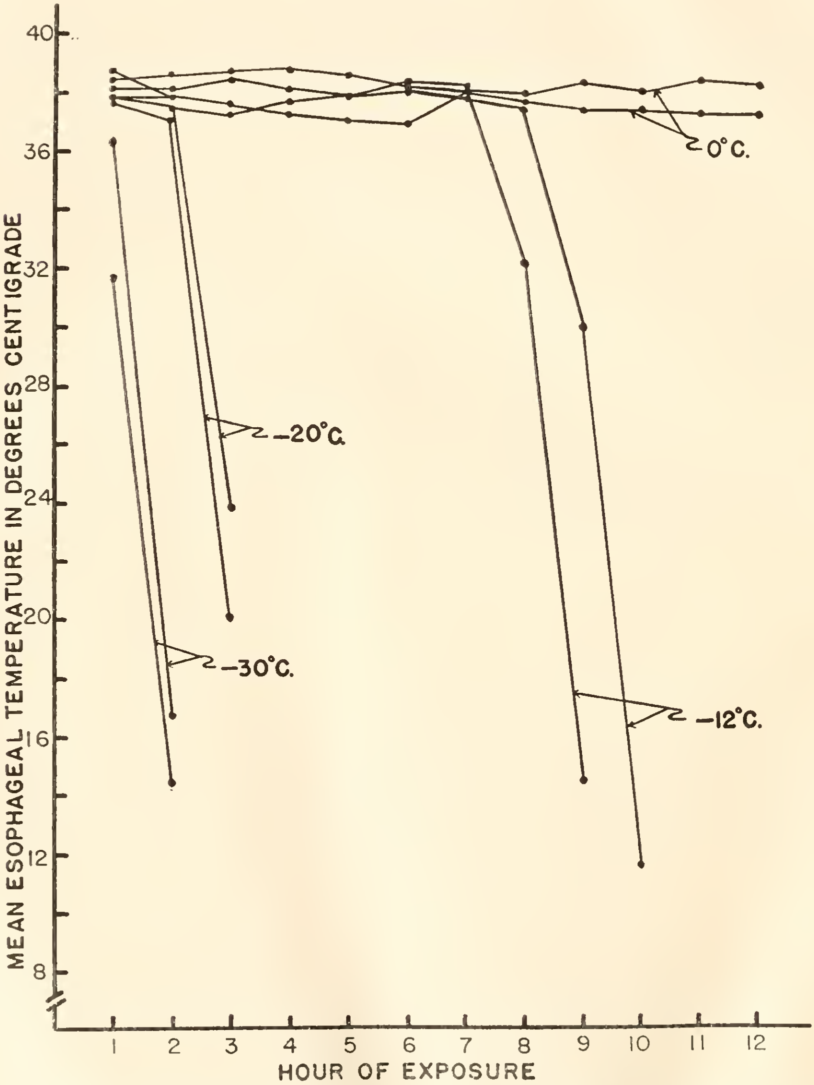


FIGURE 1. Mean esophageal temperatures of eight adult *Peromyscus leucopus noveboracensis* subjected to cold stress (0° C. to − 30° C.) when deprived of food and water.

with and without food and water. Continuous measurements of subcutaneous temperature were made, while esophageal temperatures were recorded within a 10-minute period outside the low temperature chamber after which the animals were again placed at low temperature. The possibility exists that this disturbance may have significantly altered the esophageal temperature readings. The esophageal temperature for each individual was determined at room temperature before subjecting it to lower temperatures. The mean temperature after the first hour of exposure was then averaged with the mean esophageal temperature at room temperature to give the mean temperature during the hour. The mean temperature at the end of each successive hour of exposure was then averaged with that at the end of the preceding hour in a similar fashion. Average subcutaneous temperatures were computed from automatically recorded subcutaneous temperatures for each hour of exposure. Plus and minus variations, which were of the order ± 0.5° C., tend to cancel each other out so that these averages are considered to be quite accurate.

Animals which were used more than once were kept at room temperatures for one to three days before exposing them to low temperatures again. None of the animals were exposed to low temperatures for more than 12 hours at a time, and those animals which were exposed to temperatures below 0° C. were held at room temperature for at least two days before they were again subjected to low temperature.

With but two exceptions, all individuals which were provided with food and water maintained quite uniform subcutaneous and esophageal temperatures during the entire 12-hour period of exposure at room temperatures down to about − 25° C. At temperatures around − 30° C. the mice in nearly all cases became hypothermic before the end of the first hour of exposure. When food and water were withheld the mice maintained constant temperatures during the 12-hour period only at air temperatures above 0° C. (Tables III and IV). Figure 1 shows the relationship between esophageal temperature, air temperature, and length of exposure at various degrees of low temperature with food and water withheld. Essentially the same relationship holds for subcutaneous temperatures of animals exposed to low temperature in the absence of food and water.

Some mice showed a very gradual rise in the mean body temperature during initial exposure to cold. This rise in body temperature was probably associated with an elevation of metabolism caused by exposure to low temperature as reported by a number of investigators (Adolph, 1950; Adolph and Lawrow, 1951; Benedict and MacLeod, 1929; Bodansky and Duff, 1936; Gosselin, 1949; Hart, 1950; Horvath *et al.*, 1938; Penrod, 1949; and others).

## DISCUSSION

When subjected to sufficiently low air temperature all mammals will eventually undergo a lowering of body temperature from which recovery is possible if the body temperature stays within the animal's tolerable range of hypothermia. The limits of this range vary with the duration and severity of the low temperature to which the mammal is exposed. Determinations of normal body temperatures and lower lethal temperatures for several species of mammals (Haterius and Maison, 1948; Luyet and Gehenio, 1940; Scott and Bazett, 1941; Wislocki, 1933) show that the tolerable range of hypothermia is often wide, ranging from about 13° to 37° C. For most species of mammals, including the white-footed mouse, *Peromyscus leucopus,* the tolerable range is somewhere between 20° and 25° C.

The lower lethal limits of body temperature in different species of rodents vary considerably. Hart (1951a) recorded lethal colonic temperatures from 11 deer mice, *Peromyscus maniculatus,* which ranged from 0.6° to 13.9° C. with an average of 9.2° C. The mean lethal temperature of 4.6° C. for *Peromyscus leucopus* determined in this study is considerably lower although the criteria of lethality may have varied. A Palestinian mouse, *Meriones tamaricinus,* was found to have a lower lethal temperature of about 13° C. (Bodenheimer, 1949). Lethal body temperatures of 8 white mice averaged 10° C. (Hart, 1951a). Lower lethal limits for the white rat (Adolph, 1948) and the guinea pig (Gosselin, 1949) are somewhat higher than for mice, while that of the hamster (Adolph and Lawrow, 1951) is considerably lower.

Species differences and differences in methods of measuring the lethal point undoubtedly account for much of the variation in lower lethal body temperatures of similar sized rodents. Other factors which may alter the lethal temperature are sex, amount of activity, nutritive state, relative humidity and previous acclimation to high or low temperatures. Thus the range of tolerance may vary to some extent depending upon the conditions of the experiment.

The range of tolerance above average body temperatures for most of the smaller species of rodents appears to be about the same. Data on normal body temperatures and upper lethal temperatures for the rabbit and dog (Heilbrunn, 1943; Wislocki, 1933) give tolerable ranges of hyperthermia from 4.0° to 7.5° C. The range is somewhat wider for the echidna and sloth (Britton and Atkinson, 1938; Enders and Davis, 1936; Martin, 1902, 1930; Morrison, 1945; Sutherland, 1897) in which it is between 5° and 9° C.

It seems evident from the present study that an adequate supply of food and water is important in avoidance of hypothermia at low temperatures. In addition, behavior responses such as huddling, burrowing and nest-building enable small mammals to avoid hypothermia at very low ambient temperatures (Kinder, 1927; Richter, 1927, 1942; Scholander, Walters, Hock and Irving, 1950; Sealander, 1952).

Behavior adjustments are possibly of even greater importance in avoidance of hyperthermia at high ambient temperatures. These include postural adjustments to expose greater body surface, spreading of saliva on the fur (Herrington, 1940, and this study) and decreases in amount of motor activity. According to Sumner (1925) small desert mammals have no special ability to resist high temperatures but are nocturnal in activity and remain in their burrow system during the hotter part of the day. Adjustments of this sort are more easily made than changes in the animal's energy balance.

## SUMMARY

1. Observations were made on behavior responses and body temperatures of white-footed mice, *Peromyscus leucopus noveboracensis,* subjected to ambient temperatures ranging from − 35° C. to + 50° C.

2. Behavior responses to low temperature included gnawing, grooming movements, running, rapid breathing and shivering. Motor activity was greatest upon

initial exposure to cold and at moderately low temperatures (0° to − 20° C.) and decreased almost entirely at lower temperatures (− 20° to − 35° C.). Behavior responses to high temperatures involved a decrease in activity and assumption of postures which exposed the maximum body surface area. Salivation occurred quite freely at temperatures between 40° and 50° C.

3. The lowest minimal recoverable body temperature observed was 10° C. This was an esophageal temperature when the mouse was exposed to air temperatures ranging from − 23° C. to − 12° C. over a period of 65 minutes. Activity was minimal during the entire period of exposure.

4. The mean lower lethal body temperature (esophageal) was 4.6° + 0.5° C. (15 determinations). Lower lethal body temperatures ranged from 3.5° to 5.0° C. The lower lethal body temperatures were determined at ambient temperatures ranging from − 12° to − 35° C. and the duration of exposure before death occurred varied from 45 minutes to 8.5 hours. There was, however, no apparent correlation between the lower lethal body temperature and the duration of exposure or ambient temperature. Upper lethal body temperatures of two mice were between 43° and 44° C.

5. At room temperatures esophageal temperatures ranged from 35.5° to 40.5° C. with a mean of 38.1° C. for males, and from 35.4° to 40.5° C. with a mean of 37.6° C. for females. The difference between sexes may not be significant.

6. Differences between esophageal and subcutaneous body temperatures of individual mice ranged from 0.4° to 1.7° C. The mean difference of 1.2° C. for seven mice remained the same under light anaesthesia although both esophageal and subcutaneous temperatures were subnormal.

7. Body temperatures of mice supplied with food and water remained rather constant when the mice were subjected to cold stress, except at ambient temperatures around − 30° C. When food and water were withheld all mice became hypothermic within 12 hours at all temperatures below − 1° C. These findings indicate that ordinary low temperatures may not be critical for small mammals in nature unless they are combined with lack of food, water and possibly shelter. Behavior responses such as huddling, burrowing and nest-building may give enough additional protection to offset most of the adverse effects of low air temperatures.

## LITERATURE CITED

Adolph, E. F., 1948. Lethal limits of cold immersion in adult rats. *Amer. J. Physiol.*, **155**: 378–387.

Adolph, E. F., 1950. Oxygen consumptions of hypothermic rats and acclimatization to cold. *Amer. J. Physiol.*, **161**: 359–373.

Adolph, E. F., 1951. Responses to hypothermia in several species of infant mammals. *Amer. J. Physiol.*, **166**: 75–91.

Adolph, E. F., and J. W. Lawrow, 1951. Acclimatization to cold air; hypothermia and heat production in the golden hamster. *Amer. J. Physiol.*, **166**: 62–74.

Barbour, H. G., E. A. McKay and W. P. Griffith, 1943. Water shifts in deep hypothermia. *Amer. J. Physiol.*, **140**: 9–19.

Barbour, H. G., E. A. McKay and W. P. Griffith, 1944. An approach to the use of drugs in hypothermia. *Yale J. Biol. Med.*, **16**: 231–238.

Benedict, F. G., and Gr. MacLeod, 1929. The heat production of the albino rat. I. Technics, activity control, and the influence of fasting. II. Influence of environmental temperature, age and sex; comparison with the basal metabolism of man. *J. Nutrit.*, **1**: 348–398.

Bierens de Haan, J. A., 1922. Die Körpertemperatur junger Wanderratten (*Mus decumanus*) und ihre Beeinflussung durch die Temperatur der Aussenwelt. *Arch. f. Entw.*, **50**: 1–12.

Bodansky, M., and V. B. Duff, 1936. Nitrogen and creatine metabolism in relation to environmental temperature and thyroid function. *Endocrinology*, **20**: 822–830.

Bodenheimer, F. S., 1949. Ecological and physiological studies on some rodents. *Physiol. Comp. et Oecologia*, **1**: 376–389.

Britton, S. W., 1922. Effects of lowering the temperature of homoiothermic animals. *Quart. J. Exp. Physiol.*, **13**: 55–68.

Britton, S. W., and W. E. Atkinson, 1938. Poikilothermism in the sloth. *J. Mammalogy*, **19**: 94–99.

Britton, S. W., and R. F. Kline, 1939. Emotional hyperglycemia and hyperthermia in tropical mammals and reptiles. *Amer. J. Physiol.*, **125**: 730–734.

Bullough, W. S., 1949. The effects of high and low temperatures on the epidermal mitotic activity of the adult male mouse, *Mus musculus* L. *J. Exp. Biol.*, **26**: 76–82.

Enders, R. K., and D. E. Davis, 1936. Body temperatures of some Central American mammals. *J. Mammalogy*, **17**: 165–166.

Frings, H., and M. Frings, 1952 Audiogenic seizures in the laboratory mouse. *J. Mammalogy*, **33**: 80–87.

Fuller, F. D., and W. A. Hiestand, 1947. Heat death temperatures and normal body temperatures of birds and mammals. *Turtox News*, **25**: 148–149.

Gaalaas, R. F., 1945. Effect of atmospheric temperature on body temperature and respiration rate of Jersey cattle. *J. Dairy Sci.*, **28**: 555–563.

Gelineo, S., 1934. Influence du milieu thermique d'adaptation sur la thermogénèse des homéothermes. *Ann. Physiol. et Physicochim. Biol.*, **10**: 1083–1115.

Gelineo, S., 1940. Sur la thermoregulation et la thermogénèse du rat durant un sejour prolongue aux températures provoquant l'hyperthermie. *Bull. Acad. Sci. Math. et Naturelles, B. Sci. Nat. No. 6, Acad. Roy. Serbe, 149–184.*

Giaja, J., and S. Gelineo, 1933. Sur la résistance au froid de quelques homéothermes. *Arch. Internat. Physiol.*, **37**: 20–34.

Gosselin, R. E., 1949. Acute hypothermia in guinea pigs. *Amer. J. Physiol.*, **157**: 103–115.

Hart, J. S., 1950. Interrelations of daily metabolic cycle, activity, and environmental temperature of mice. *Canad. J. Res., D*, **28**: 293–307.

Hart, J. S., 1951a. Calorimetric determination of average body temperature of small mammals and its variation with environmental conditions. *Canad. J. Zool.*, **29**: 224–233.

Hart, J. S., 1951b. Average body temperature in mice. *Science*, **113**: 325–326.

Haterius, H. O., and G. L. Maison, 1948. Experimental hypothermia and rewarming in the dog: Recovery after severe reduction in body temperature. *Amer. J. Physiol.*, **152**: 225–232.

Heilbrunn, L. V., 1943. An outline of general physiology. W. B. Saunders Co., Phila. Second Edition.

Herrington, L. P., 1940. The heat regulation of small laboratory animals at various environmental temperatures. *Amer. J. Physiol.*, **129**: 123–139.

Hock, R. J., 1951. The metabolic rates and body temperatures of bats. *Biol. Bull.*, **101**: 289–299.

Horvath, S. M., F. A. Hitchcock and F. A. Hartman, 1938. Response to cold after reduction of adrenal tissue. *Amer. J. Physiol.*, **121**: 178–184.

Kendeigh, S. C., 1945. Body temperatures of small mammals. *J. Mammalogy*, **26**: 86–87.

Kinder, E. F., 1927. A study of the nest-building activity of the albino rat. *J. Exp. Zool.*, **47**: 117–161.

Kredl, F. E., 1928. Notes on the temperature of the sloth. *J. Mammalogy*, **9**: 48–51.

Luyet, B. J., and P. M. Gehenio, 1940. Life and death at low temperatures. Biodynamica, Normandy, Missouri.

Martin, C. J., 1902. Thermal adjustment and respiratory exchange in monotremes and marsupials—A study in the development of homoiothermism. *Phil. Trans. Roy. Soc. Lond., B*, **195**: 1–37.

Martin, C. J., 1930. Thermal adjustment of man and animals to external conditions. *Lancet*, **2**: 561–566, 617–620, 673–678.

Morrison, P. R., 1945. Acquired homiothermism in the pregnant sloth. *J. Mammalogy,* **26**: 272–275.

Morrison, P. R., 1946. Temperature regulation in three Central American mammals. *J. Cell. Comp. Physiol.,* **27**: 125–138.

Morrison, P. R., and F. A. Ryser, 1951. Heterothermism in the deer mouse, *Peromyscus leucopus. Anat. Rec.,* **111**: 513–514.

Penrod, K. E., 1949. Oxygen consumption and cooling rates in immersion hypothermia in the dog. *Amer. J. Physiol.,* **157**: 436–444.

Przibam, H., 1917. Direkte Temperaturabhängigkeit der Körperwarme bei Ratten (*Mus decumanus* und *M. rattus*). *Arch. f. Entw.,* **43**: 37–46.

Reeder, W. G., and R. B. Cowles, 1951. Aspects of thermoregulation in bats. *J. Mammalogy,* **32**: 389–403.

Richter, C. P., 1927. Animal behavior and internal drives. *Quart. Rev. Biol.,* **2**: 307–343.

Richter, C. P., 1942. Physiological psychology. *Ann. Rev. Physiol.,* **4**: 561–574.

Scholander, P. F., V. Walters, R. Hock and L. Irving, 1950. Body insulation of some arctic and tropical mammals and birds. *Biol. Bull.,* **99**: 225–236.

Scholander, P. F., R. Hock, V. Walters, F. Johnson and L. Irving, 1950. Heat regulation in some arctic and tropical mammals and birds. *Biol. Bull.,* **99**: 237–258.

Scholander, P. F., R. Hock, V. Walters and L. Irving, 1950. Adaptation to cold in arctic and tropical mammals and birds in relation to body temperature, insulation, and basal metabolic rate. *Biol. Bull.,* **99**: 259–271.

Schwabe, E. L., E. E. Emery and F. R. Griffith, Jr., 1938. The effect of prolonged exposures to low temperature on the basal metabolism of the rat. *J. Nutrit.,* **15**: 199–210.

Scott, J. C., and H. C. Bazett, 1941. Temperature regulation. *Ann. Rev. Physiol.,* **3**: 107–130.

Sealander, J. A., 1951a. Survival of Peromyscus in relation to environmental temperature and acclimation at high and low temperatures. *Amer. Midl. Nat.,* **46**: 257–311.

Sealander, J. A., 1951b. A technique for continuous recording of subcutaneous temperatures of wild mice. *J. Mammalogy,* **32**: 122–123.

Sealander, J. A., 1952. The relationship of nest protection and huddling to survival of Peromyscus at low temperature. *Ecol.,* **33**: 63–71.

Seath, D. M., and G. D. Miller, 1946. The relative importance of high temperature and high humidity as factors influencing respiration rate, body temperature, and pulse rate of dairy cows. *J. Dairy Sci.,* **29**: 465–472.

Sumner, F. B., 1913. The effects of atmospheric temperature upon the body temperature of mice. *J. Exp. Zool.,* **15**: 315–378.

Sumner, F. B., 1925. Some biological problems of our southwestern deserts. *Ecol.,* **6**: 352–371.

Sutherland, A., 1897. The temperature of reptiles, monotremes and marsupials. *Proc. Roy. Soc. Victoria (n.s.),* **9**: 57–67.

Wade, O., 1930. The behavior of certain spermophiles with special reference to aestivation and hibernation. *J. Mammalogy,* **11**: 160–188.

Wislocki, G. B., 1933. The location of the testes and body temperature in mammals. *Quart. Rev. Biol.,* **8**: 385–396.

Wislocki, G. B., and R. K. Enders, 1935. Body temperature of sloths, anteaters, and armadillos. *J. Mammalogy,* **16**: 328–329.

# CHEMOTROPISM IN RHIZOPUS NIGRICANS. II. THE ACTION OF PLANT JUICES

DAVID R. STADLER

*Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena 4, California*

Parasitic fungi invade the tissues of their host plants by sending their growing hyphal tips through the stomata of the leaves or by actually piercing the epidermis. To pass through a stoma, the hypha must first grow to a particular point on the leaf surface. To penetrate the epidermis, it must break through a barrier. Investigators have long believed that such non-random patterns of growth must involve some directive influence by the host plant. Such influence seems even more necessary in view of the high specificity shown by some parasites—they will invade the tissues of only a particular kind of host. The question of how a host plant could direct the growth of its parasite led to some early studies of chemical tropism in fungi.

The first work dealing directly with this problem was that of Miyoshi (1894), in which he sowed spores of several molds on the under surface of Tradescantia leaves which had been injected with various chemical substances. These substances presumably diffused out through the stomata and onto the surface. He reported that when the leaves contained proper concentrations of certain nutrients (particularly sugars and meat extracts), the hyphae grew directly to and through the stomata. On leaves injected only with water, he found the hyphae to grow in random directions, while if the leaves contained harmful substances (acids, alkali, alcohol and certain salts), growth was oriented away from the stomata. He observed the same tropic responses when he sowed the spores on mica plates with pin holes in them, the substance under test being in a gelatin layer in contact with the opposite face of the plate. Thus Miyoshi concluded that fungi exhibit an extensive (and extremely useful) chemotropic sensitivity, guiding them to supplies of nourishment and away from damaging materials.

The case for chemotropic control of parasitic molds was expanded by Massee (1905). He confirmed Miyoshi's finding that various fungi grow tropically toward sugars. But he found obligate parasites to be attracted only by decoctions from their own hosts. He believed facultative parasites to be attracted by sugar, but repelled by certain other substances, which prevented them from invading certain types of hosts (thus he said Botrytis failed to grow into green apples because of a negative tropic sensitivity to malic acid). Massee concluded that the specificity of parasitism was based completely on chemotropism. His findings have not been confirmed in any other work, to our knowledge. Certain substances for which he reported strong chemotropic action have been tested on Rhizopus in the course of the current study, but always with negative results.

The findings of Miyoshi were checked by Clark (1902), using the injected Tradescantia leaves, but with quite different results. He wished particularly to test the suggestion of Swingle (1896) that sprays of copper compounds protect host plants through a negative chemotropic action on parasitic fungi. He concurred with Miyoshi in the finding that the hyphae grew into Tradescantia stomata toward sugars, but he found them to grow in just as readily if the leaf had been injected with plain water or even with toxic copper salts. Likewise, on mica plates, the hyphae grew into the hole regardless of what substance was on the other side. The only condition which prevented growth toward the hole was the presence of germinating spores in the layer on the other side. He concluded that the germ tubes growing toward the holes when no spores were present on the other side must have tropic sensitivity to some product of the mold metabolism and must be growing *away* from higher concentrations of this material. This growth behavior has been called the "staling reaction," as it is growth away from regions of stale medium. Clark's conclusion that molds have a negative tropic sensitivity to some product of their own metabolism has been adequately confirmed by later studies (Fulton, 1906; Graves, 1916; Stadler, 1952).

There has been some disagreement as to whether sugars and other nutrients exercise a positive chemotropism on molds. Miyoshi (1894) reported pronounced turning of the hyphal tips toward concentrations of various nutrients, but Clark (1902) maintained that the oriented growth was caused only by the negative tropic effect of the staling factor, and that hyphae could grow just as readily into water or harmful substances as into nutrients. This was the view also of Fulton (1906), who looked for tropic action by a large number of substances on eight different molds; he detected no positive tropism and concluded that orientation was based solely on the staling reaction. Graves (1916) recognized that the staling reaction caused the most marked orientation, but he reported that hyphae turned more pronouncedly toward a sugar-containing medium than toward plain water; thus, he concluded that sugar does exercise an attraction, but that it is normally masked by the stronger tropic action of the staling substance.

Some of the experiments on sugars were repeated during the present study in an attempt to determine whether or not there was any tropic action by these substances. The interpretation of such studies is complicated by the fact that staling-substance production (and thus orientation caused by the staling reaction) varies with sugar concentration. When the experiments were designed in a way to minimize variation in the staling reaction, the results gave some indication of a very slight attraction by glucose and by sucrose, but they were not conclusive.

Graves reported that turnip juice medium had a much more powerful positive tropic effect than sugars, though again, not as strong as the staling reaction. The turnip juice finding was reminiscent of the report of Massee (1905) and other early suggestions that tropic attraction by plant juices might be responsible for host penetration by parasitic fungi.

In the present study the tropic action of turnip juice and other plant materials on germinating spores of *Rhizopus nigricans* has been extensively studied. These materials cause striking orientation, but further investigation of this phenomenon has led us to the conclusion that this orientation is not the result of a direct tropic action on the mold by the plant juices.

## METHODS

The method used in most of the experiments is the same as described previously (Stadler, 1952); plates of plastic coverslip material with circular holes drilled in

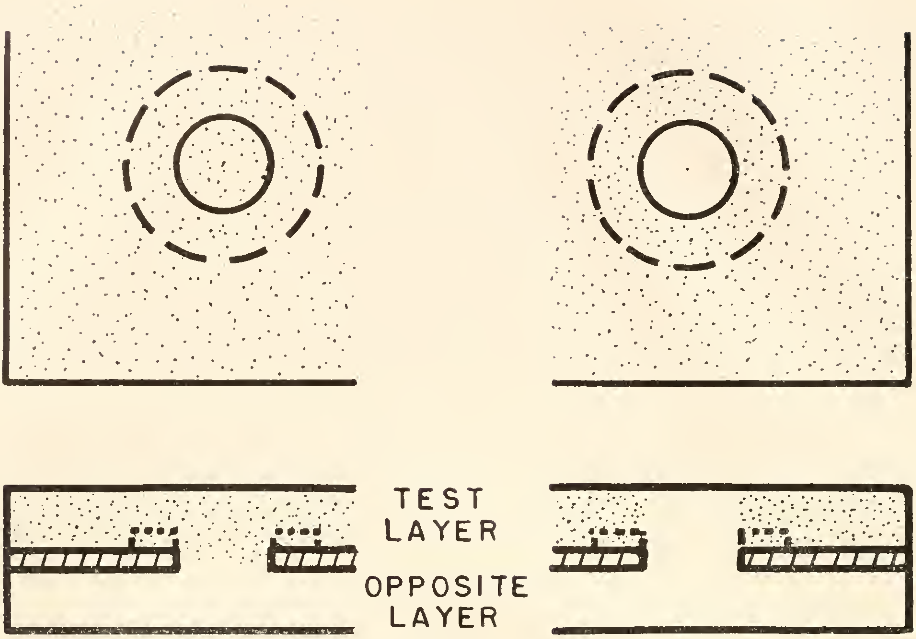


FIGURE 1. Enlarged representations of the corner of a plate showing one hole in face view and in profile. On the left is the plate as prepared in the ordinary tests. The dots represent the spores of the test layer, and the broken line shows the limits of the test region. The picture on the right shows a preparation made by the "thumb tack" method, whereby no spores are present within or above the hole and the test region becomes the edge of the population.

them are used. A layer of agar medium is placed on each side of the plate; the agar layers are in contact with each other only within the holes. Any material contained in one layer but not in the other will tend to diffuse through the hole, setting up a concentration gradient in the region in and near the hole. One of the layers, in every case, contains a suspension of spores of Rhizopus. This is the "test layer." The direction of growth is studied of the spores in the test layer in the region immediately around the hole. The other layer is called the "opposite layer" and may or may not contain spores, depending on the experiment being done. The preparations are incubated for eight and one half hours at 28° C. and then fixed and stained. Camera lucida drawings are made of the spores in each of the "test regions" (washer-shaped region circumscribing the hole, Fig. 1), and the directions of growth are measured. The angle measured is the direction of growth of the germinating spore with respect to the direction of the chemical gradient. Any gradient set up between the two layers must be oriented directly out from the center of the hole. When a germ tube grows directly toward the center of the hole, the angle recorded is zero; when it grows directly away from the hole, the angle is 180 degrees.

If the germ tubes of spores in a particular experiment are growing in random directions, then the average of the angles for all the spores in a test region should be about 90 degrees. This is the result when we do an experiment with spores in nutrient medium in the test layer and the same concentration of spores in the same medium in the opposite layer. (The synthetic medium used in these experiments contained 1% glucose, $10^{-2}$ $M$ aspargine, $10^{-2}$ $M$ $KH_2PO_4$, and $2 \times 10^{-3}$ $M$ $MgSO_4$.) When a plate is prepared with spores in the nutrient medium in the test layer, and the opposite layer is made up with the same medium (or even water agar) but with no spores, then the spores in the test regions grow toward the holes. If the test layer contains 120 spores per mm.$^3$, the average angle will be 40–45 degrees (Fig. 2-A). This is the staling reaction—growth away from populated regions, away

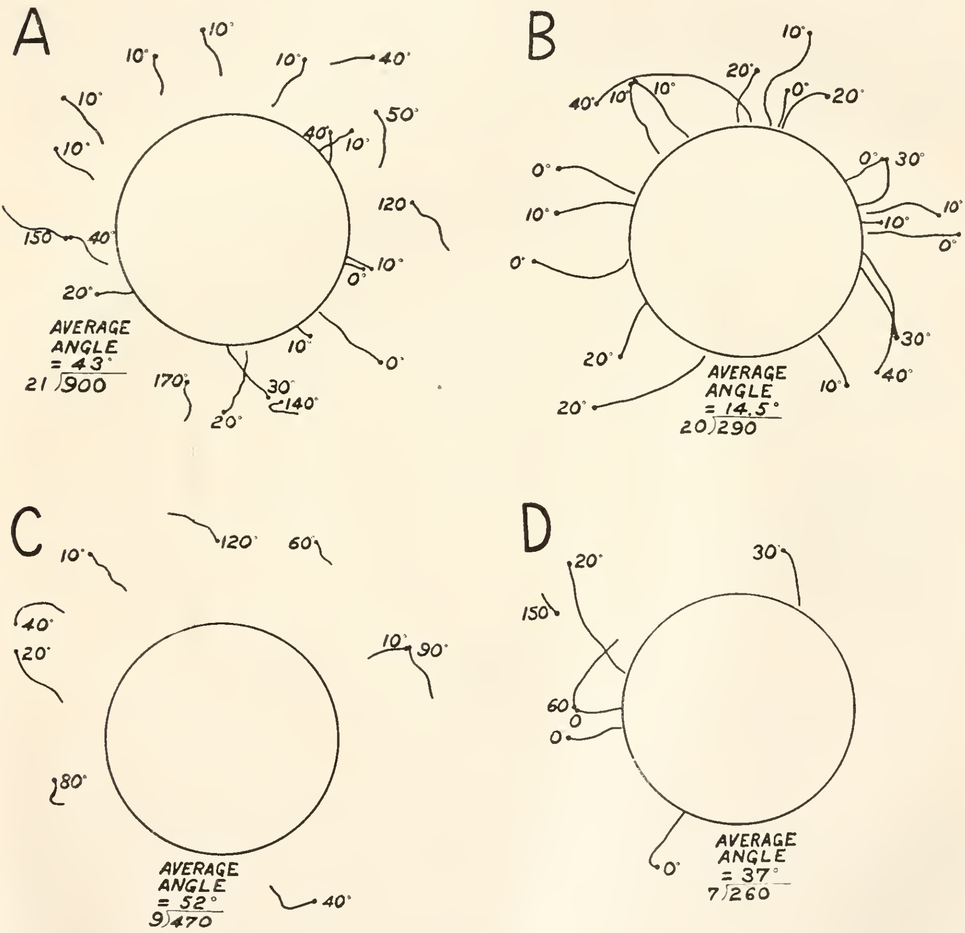


FIGURE 2. Camera lucida drawings of test regions from experimental plates showing the improvement of orientation elicited by plant juices in the opposite layer. *A:* 120 spores per mm.$^3$ in the synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *B:* 120 spores per mm.$^3$ in synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer; *C:* 40 spores per mm.$^3$ in synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *D:* 40 spores per mm.$^3$ in the synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer.

from concentrations of the unknown metabolic product (or products) called the staling substance.

## The "Attraction" of Plant Juices

If turnip juice is mixed into the non-spore opposite layer of a plate with spores in synthetic medium in the test layer, all the spores in the test region will germinate and grow toward the hole. The orientation is much more marked than when synthetic medium is used in the non-spore opposite layer (Fig. 2-A, B); in that case the average angle was 40–45 degrees; with turnip juice in the opposite layer it is 10–20 degrees.

This same "attraction" is elicited by several other natural mixtures from plant sources. Yeast extract in the opposite layer causes striking orientation toward the hole. Tomato juice does the same, as does a decoction of elm leaves. A strain of Penicillium has been isolated which imparts this property to the synthetic medium when grown in that medium for one week. (This strain has been identified by Dr. Kenneth B. Raper as *Penicillium spinulosum* Thom.) The young growing mycelium of Rhizopus itself if heated to 60° C. for one hour, releases into the medium around it a material with this same effect. These "attractants" from various sources are all alike in that they are water-soluble and heat stable, and all evoke the same marked orientation of Rhizopus on the plates used in this study. Furthermore, they are alike in that there is evidence in each case that the material does not exercise a true chemotropic attraction on the Rhizopus spores.

Table I

*Comparison of "attraction" effect in high and low spore concentrations*

| "Attractant" | Spore concentration in test layer (per mm.$^3$) | Average Angle | | Average improvement of orientation (in degrees) by "attractant" |
|---|---|---|---|---|
| | | With non-spore synthetic medium in opposite layer | With "attractant" added to nonspore opposite layer | |
| Elm leaf decoction | 120 | 45 degrees | 17 degrees | 28 |
| | 40 | 54 degrees | 38 degrees | 16 |
| Cooked Rhizopus (medium in which Rhizopus has been grown and heated to 60% C. for one hour before being filtered off) | 120 | 47 degrees | 27 degrees | 20 |
| | 40 | 55 degrees | 46 degrees | 9 |
| Yeast extract | 120 | 42 degrees | 22 degrees | 20 |
| | 40 | 57 degrees | 42 degrees | 15 |

Note: Each average angle given in this table is the mean value of four test regions.

The suspicion arose that these materials might not exert a simple attraction on Rhizopus when it was noted that a preparation which markedly improved the orientation on plates with high concentrations of spores in the test layer had much less effect on orientation on plates with low spore concentrations (Table I, Fig. 2).

This suggested that the action of these materials might be somehow related to the staling reaction, since the intensity of this reaction varies with spore concentration. If the material were a true attractant, it should act on spores in its presence regardless of the intensity of the staling reaction.

Orientation, in these experiments, is normally limited by a shortcoming of the method. The strongest orientation elicited by the staling reaction should be shown by the spores on the *edge* of the populated region, as this is where the concentration of staling substance is diminishing most markedly. The spores in the test regions of these plates are not on the edge of the population, but near the edge. There are always some spores *within* the hole—this is the edge of the population mass; the test region is back from the edge (Fig. 1). When an experiment is done with a sparse population of spores in the test layer, there are very few spores within the hole, and these have a minimal effect on the amount of orientation. But in heavy populations the spores within the hole become important, as there are enough of them to produce considerable staling substance and seriously limit the orientation in the test region. The extent of orientation depends upon the steepness of the concentration gradient of staling substance in the test region. If it is being produced in large amounts not only on the side away from the hole, but also on the side toward (in) the hole, then there cannot be a sharp gradient. That this is the true situation is shown by a series of experiments with various concentrations of spores in synthetic medium in the test layers and non-spore synthetic medium in the opposite layers. Orientation improves with increasing spore concentration up to about 80 spores per mm.$^3$ (Fig. 3). At greater spore concentrations the orientation of spores in the test region does not improve; higher levels of staling substance away from the hole are being matched by higher levels within the hole. (The same limitation applies to the method used in the studies of Graves (1916) and earlier workers, who did their experiments on mica plates with pin holes in them.)

If a plate is prepared with one of the "attractants" in the opposite layer, this material is in contact with the test layer within the hole, and during the incubation it diffuses out through the hole towards the test region. When it was found that these "attracting" materials showed pronounced effects only on plates with high spore concentrations in the test layer, it was suspected that they might function by inactivating staling substance in their presence. This would "flush" the staling substance within the hole, and permit a steep concentration gradient of this material to arise in the test region. The hypothesis is advanced, then, that these "attracting" materials act not by any true positive chemotropic effect on the spores, but only by inactivating a substance which does have a true tropic action.

As a further check of this hypothesis, test plates were set up with no spores in or above the hole. In this situation the test region becomes the very edge of the population (Fig. 1). This is accomplished by means of thumb tacks with the same bore as the holes in the plastic plates. The tacks are inserted in the holes before the test layer is poured, and after it has solidified they are removed, leaving a cylindrical hollow above each hole. When the plate is placed on the liquid, non-spore agar medium of the opposite layer, this material fills these hollows before it solidifies. When the growth on these preparations is examined, the test spores are found to show excellent orientation (15–20 degrees average), regardless of whether an "attractant" is present in the opposite layer or not. Thus the pronounced effect of the "attractants" seems to be directly dependent on the presence of spores within the hole.

A simpler test which indicates that this is not a true attraction is done with a layer of agar medium on a glass slide. Spores are inoculated only at one end, and the hyphae grow down the length of the slide. A block of the synthetic medium agar in the region ahead of the advancing hyphae is cut out and replaced by a block of turnip juice agar. If it were a true attractant, hyphae passing nearby should turn and grow into it, but they do not.

The experiments so far described fit very well the hypothesis that the "attracting" materials act only by inactivating staling substance in their presence. However, with undiluted tomato juice agar in the opposite layers of test plates, we observe marked improvement of orientation even when there is a low concentration of spores in the test layer. The method used in this experiment is not satisfactory in that it can never tell us whether the improved orientation results from an enhanced staling reaction or from a true positive tropic effect. This is because both of these possible effects would be working in the same direction in this system, and there is thus no visible way to distinguish between them. The same ambiguity exists, to a limited extent, in the preparations with no spores within the holes, though these were designed to minimize any effect on the staling reaction. The same is true for the experiment with a block of turnip juice agar in the neighborhood of growing hyphae.

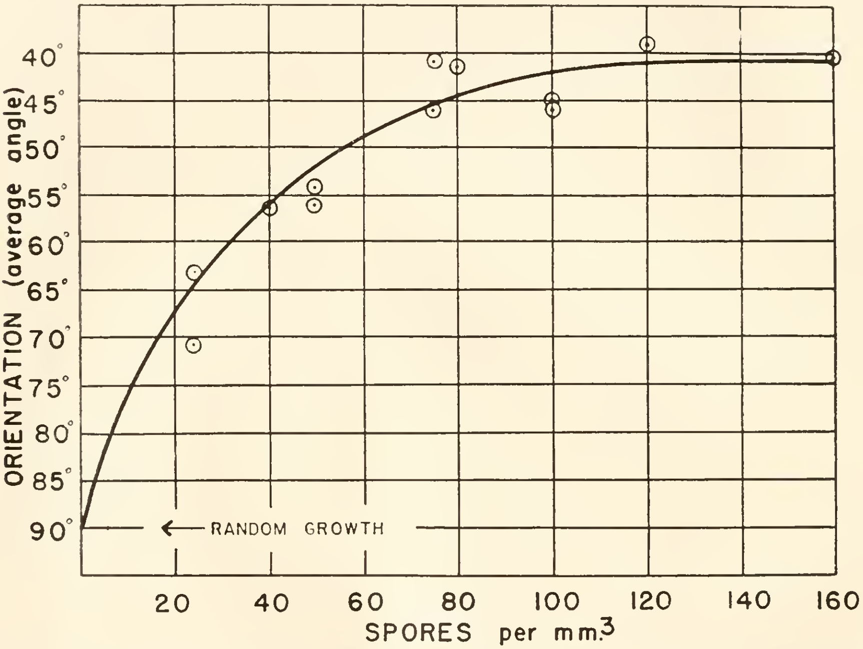


Figure 3. Orientation plotted against spore concentration for experiments with spores in synthetic medium in the test layer and non-spore synthetic medium in the opposite layer. Each point represents the average of four test regions.

A definitive test to learn the true nature of the effect can be designed by preparing the experiment in such a way that the "attractant" will be working in a direction antagonistic to the staling reaction. This is accomplished by putting the "attractant" material in the test layer with the spores, while the opposite layer contains non-spore synthetic medium. In this situation, the staling reaction tends to make the spores in the test region orient toward the hole, while the "attractant" tends to orient them away from the hole. If the experiment is prepared so that the staling reaction is very weak (low spore concentration), and the attraction is very strong (undiluted tomato juice), then we can tell whether it is a true attractant or a staling substance inactivator. If it is a true attraction, then it should completely outweigh the staling reaction in this situation and cause marked orientation away from the hole. If it works only by inactivating staling substance, then the most it can do is obliterate the staling reaction and cause random growth; 90 degrees becomes the limiting average angle. Table II shows the latter to be the case. With concentrated tomato juice on a series of plates with very low spore concentration, the average angle is below 90 degrees. With turnip juice, the result is the same. This is good evidence, then, that tomato juice and turnip juice, which showed the most marked effects of any of the materials in the previous tests, are not true chemotropic agents.

TABLE II

*Orientation of spores in plant juice with synthetic medium in the opposite layer*

| "Attractant" in test layer | Spore concentration in test layer (per mm.$^3$) | Number of test regions studied | Total no. of hyphae | Total angle | Average angle |
|---|---|---|---|---|---|
| Tomato juice | 25 | 16 | 74 | 6000 degrees | 81 degrees |
| Turnip juice | 35 | 15 | 105 | 8950 degrees | 85 degrees |

Note: In the experiments reported in this table, all opposite layers contained non-spore synthetic medium.

Attempts at purification of the active material by extraction of yeast extract with a number of solvents (methanol, propanol, butanol and chloroform) have been unsuccessful. Paper chromatograms of yeast extract with a butanol-acetic acid mixture as the moving solvent affected some separation of components but did not result in any appreciable purification of the active material. Further chemical studies are projected with other solvents and perhaps other of the source materials.

The presence of a dialysis membrane between the two agar layers on the test plate does not hinder the effect of the "attractant" (from the Penicillium medium), but there is indirect evidence that the active material is slow-diffusing. The "attraction" phenomenon is qualitatively unchanged at any acidity permitting growth of the mold (pH 3 to pH 7).

## DISCUSSION

The experiments which were done to determine the nature of the action of plant juices on orientation of the growth of Rhizopus spores demonstrated that the intensity of the plant juice effect varies with the amount of growing mold in its presence. The hypothesis has been advanced that the plant juices affect orientation by inactivating staling substance in their presence. It is also possible that they act by preventing the production of staling substance. A third possibility which fits the experimental findings equally well is that a true positive tropic agent is formed by the combination of a plant juice ingredient with some product of the mold's metabolism. Not knowing the chemical nature of the active plant juice material, it is difficult to design experiments which would discriminate between these various hypothetical mechanisms.

The present study does not enable us to evaluate the significance in nature of the tropic action of plant juices. Although these materials do not exercise direct attraction on the hyphae (of Rhizopus, at least), they do act in a way which could facilitate entrance into the stomata by germ tubes of spores germinating on the leaf surface. To understand the part played by chemical tropism in host-parasite relationships, a careful study should be made of the tropic sensitivities of a series of parasitic molds. If their tropic responses are similar to those of Rhizopus, then it does not seem possible that host specificity could be based on unique chemotropic sensitivities, as suggested by Massee (1905). In view of the many instances of specific growth factor requirements of molds which have been demonstrated in recent years, it appears more probable that this is the basis of host-parasite specificity.

## Summary

Turnip juice and several other plant materials exert what appears to be a strong tropic attraction on the germinating spores of *Rhizopus nigricans* when tested on the type of experimental plates used in this and earlier studies. However, evidence is presented which demonstrates that the plant juices do not exercise a direct tropic action on the mold. It is suggested that these materials function by inactivating the staling substance (a negative tropic agent which is a normal product of the mold's metabolism).

## LITERATURE CITED

Clark, J. F., 1902. On the toxic properties of some copper compounds with special reference to Bordeaux mixture. *Bot. Gaz.*, 33: 26–48.

Fulton, H. R., 1906. Chemotropism of fungi. *Bot. Gaz.*, **41**: 81–108.

Graves, A. H., 1916. Chemotropism in *Rhizopus nigricans*. *Bot. Gaz.*, **62**: 337–369.

Massee, G., 1905. On the origin of parasitism in fungi. *Phil. Trans. Royal Soc.* (*B*), **197**: 7–24.

Miyoshi, M., 1894. Ueber Chemotropismus der Pilze. *Bot. Zeitschr.*, **52**: 1–28.

Stadler, D. R., 1952. Chemotropism in *Rhizopus nigricans:* the staling reaction. *J. Cell. Comp. Physiol.*, **39**: 449–474.

Swingle, W. T., 1896. Bordeaux mixture. Bull. No. 9, Div. Veg. Phys. & Path., U. S. Dept. Agric.

# RESPIRATORY GRADIENTS IN TUBULARIA[1]

L. C. SZE[2]

*Department of Zoology, Columbia University, New York 27, New York*

The presence of a respiratory gradient along the stem of Tubularia has been known for some time (Hyman, 1926; Barth, 1940a). It has been suggested that it is the underlying factor of the regeneration gradient (Barth, 1940b). One can easily find considerable evidence in previous works of various investigators in favor of this idea (Barth, 1940b). For instance, regeneration rates along the stem are roughly proportional to the rate of oxygen consumption and oxygen is an important factor in the rate of regeneration and in determination of the polarity of the stem. However, the evidence so far does not seem critical enough for its establishment.

More recently, Rose and Rose (1941), Miller (1942), and Goldin (1942a) have shown that a certain inhibitor (or inhibitors) of regeneration is produced by the stem. Goldin (1942a, 1942b) and Barth (1944) showed that a ratio involving the concentration of inhibitors and oxygen, $O_2/\ (\Sigma I)$, somehow determines the rate of regeneration and the polarity of the stem. Thus, another hypothesis suggested by Barth (1944) is that a gradient in the concentration of the inhibitor is the cause of polarity and of the determination of the polarity of the stem. Oxygen merely shifts the threshold of inhibition.

The purpose of the present investigation is to test which of the two hypotheses is more likely. It has been known that the regeneration polarity of Tubularia can be reversed by various methods. If the respiratory gradient is the cause of this polarity, one would expect that when we use one of the methods to reverse the polarity of the stem, the respiratory gradient should reverse its direction before the polarity.

## METHODS

There are a number of techniques which can be used for reversing the polarity of Tubularia, for instance, differential concentration of oxygen at two cut ends, ligature (Barth, 1938) etc. The method adopted for reversing the polarity in the present paper was ligature. The material used was freshly collected from Atlantic Beach, Long Island, New York City. A number of healthy stems was carefully selected. They were cut into fragments a little more than 10 mm. long. The distal ends of these fragments were then tied individually with a length of hair. Finally they were placed in sea water through which pure oxygen was bubbled, and kept at 15° C. until the initiation of measurements.

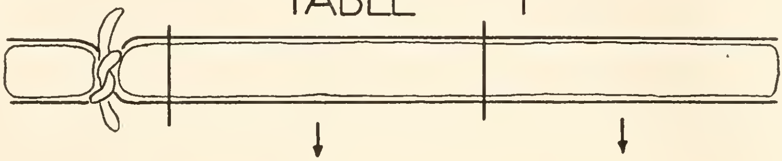
For measuring the oxygen uptake, Cartesian diver technique was used. Before the measurement of $O_2$ uptake, fragments were individually checked under a microscope to see whether they were in healthy condition and whether the primordia had been formed. Only the healthy fragments were used in the experiments. Each fragment was then cut into two pieces, the distal and proximal halves of approximately similar size (Tables I, II and III). The respiratory rates of the two pieces derived from the same fragment were then compared. All measurements were made at 25° C. It is very hard in New York City to keep the temperature below this in summer. Although this temperature is higher than normal, the rate of respiration during the period of measurement is constant in all cases. After the measurement, the pieces were carefully taken out of the divers. They were rinsed once with distilled water and were subsequently transferred onto the weighing pans. Following this, they were dried overnight in an oven at 120° C. and were then weighed on a quartz torsion balance.

---

[1] Aided by a grant from the Committee on Growth acting for the American Cancer Society, administered by Dr. L. G. Barth.

[2] Current address: The Creedmoor Institute for Psychobiologic Studies, Queens Village 27, New York.

TABLE I



| Frag. No. | Rate Distal | Rate Proximal |
|---|---|---|
| 1 | 0.76 | 0.60 |
| 2 | 0.83 | 0.56 |
| 3 | 0.76 | 1.0 |
| 4 | 0.63 | 0.53 |
| 5 | 1.0 | 0.85 |
| $\bar{X}$ | 0.80 | 0.71 |

TABLE I. The respiratory rates of the fragments of the non-regenerating stems. The rate $\times 10^{-3}$ μl./μg. dry wt./hr.

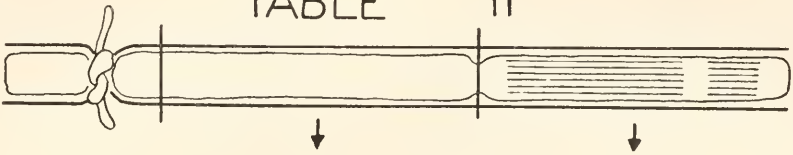
## RESULTS

In Table I, there are five experiments made on three-day old fragments that have no primordium. Four fragments show that the distal half has a higher respiratory rate than the proximal portion. One fragment gives the opposite result. On the average the distal half respires more than the proximal. These results agree with the findings of Hyman (1926) and Barth (1940a).

Four experiments have been made on the fragments which have primordia at their proximal ends and no primordia at the distal ends (ligature end). The results are summarized in Table II. From the table one can see that all the distal halves invariably show higher respiratory rates than the proximal ends, although the latter bear primordia.

In Table III, there are five experiments in which the fragments have very well-developed hydranths. The measurements were made three days after the emergence of the hydranth from the perisarc. The pieces for measurements of oxygen uptake were cut in the way shown in the figure of Table III. Only the region that was covered by the old perisarc was used for these measurements. This method of cutting was employed in order to eliminate possible variation of the thickness of the newly formed perisarc from the old one. From the table we can see that there is no apparent difference between the distal and the proximal piece.

TABLE II

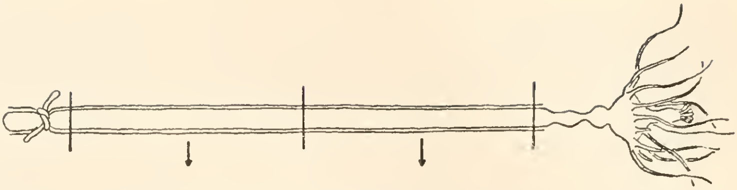


| Frag. No. | Rate Distal | Rate Proximal |
|---|---|---|
| 1 | 1.8 | 1.0 |
| 2 | 1.2 | 0.95 |
| 3 | 1.2 | 0.68 |
| 4 | 1.6 | 1.2 |
| $\bar{X}$ | 1.5 | 0.96 |

Table II. The respiratory rates of the fragments of the regenerating stems. The rate $\times 10^{-3}$ $\mu$l./$\mu$g. dry wt./hr.

From the results, it is clear that before or during the process of regeneration, there is no apparent change in respiratory gradient. Unfortunately, no running sea water was available. Although sea water was changed every day during the course of investigation, the regenerated hydranth usually dropped off the stem on the third or fourth day after emergence of the hydranth from the perisarc. However, the results on the fragments with well developed hydranths do suggest the tendency of the reversal of the respiratory gradient, since the distal and proximal pieces of such fragments respire at approximately the same rate, three or four days

TABLE III



| Frag. No. | Rate Distal | Rate Proximal |
|---|---|---|
| 1 | 0.99 | 0.71 |
| 2 | 0.71 | 0.93 |
| 3 | 0.62 | 0.66 |
| 4 | 0.78 | 0.68 |
| 5 | 0.86 | 0.86 |
| $\bar{X}$ | 0.79 | 0.77 |

TABLE III. The respiratory rates of the fragments of the polarity-reversed stems. The rate × $10^{-3}$ µl./µg. dry wt./hr.

after emergence of hydranth from perisarc. Probably, if one could keep the fragments long enough their respiratory gradient would eventually be reversed.

## DISCUSSION

There are two drawbacks concerning the present results. First, the temperature at which the experiments were made may have been too high, even though constant respiratory rates were obtained in all cases during the period of measurement. Second, although measurements of two contiguous pieces of each fragment were made, as shown in the tables, there may nevertheless have been some variation in the thickness of the perisarc, a factor which would affect the total dry weight. In other words, the respiratory gradient could possibly be due to a posterior segment containing more perisarc material and less tissue. Some preliminary experiments indicate that this might well be the case. In the following discussion we shall assume that these two drawbacks are not of great importance. A more extensive study under controlled conditions is of course necessary.

It is possible that techniques which involve keeping an animal at one temperature and measuring oxygen consumption at another may produce a differential acceleration in regions which are compared. If there is any such differential effect of temperature, it does not appear to be important. Barth (1940a) prepared his material at room temperature and measured the oxygen uptake at 18.5° – 19.0° C. His results on the respiratory gradient of the stem are in agreement with the present findings in the experiments on the stems with or without primordia.

A number of investigators have demonstrated the presence of an inhibitor of regeneration. Certain experiments have indicated that the inhibitor substance is a fairly diffusible one (Rose and Rose, 1941; Goldin, 1942a). Barth (1940a) found that the $O_2$ consumption of regenerating stems is no greater than the $O_2$ consumption of resting, non-regenerating stems. Thus, stems which were prevented from regenerating by means of ligatures at the ends consumed as much oxygen as stems which regenerated freely. These facts suggest two things: (1) the inhibitor of regeneration is either a weak respiratory inhibitor or an inhibitor not affecting respiration at all; and (2) ligatures have no or very little effect on the respiration of the stem.

It was shown in the present investigation that prior to, or during the process of regeneration, the original respiratory gradient is maintained. In view of the conclusions in the preceding paragraph, these findings really represent what had been going on in the stems before removal of the ligature and cutting. Therefore one may say from the present investigation that the formation of the hydranth is independent of the respiratory gradient. The hydranth is not always formed at the place where the highest respiratory activity exists. If a respiratory gradient were involved in regeneration, one would expect, at least during the time of regeneration, that the primordium would show a higher respiratory activity than the distal segment in the present experiments. Since the presence of an inhibitor of regeneration in the stem has been demonstrated and it has also been found that oxygen plays a very important role in regeneration, it seems most likely that the position of a future hydranth in a regenerating stem is determined by the balance between the concentration of the inhibitor of regeneration and the concentration of oxygen.

Finally, the present findings seem to suggest that the respiratory gradient of the stem is the result of the presence or the activity of a well developed hydranth.

## Summary

It was found that during regeneration of the hydranth, the original respiratory gradient of the stem is maintained. Only after the hydranth has formed has the stem a tendency to reverse its original respiratory gradient.

## LITERATURE CITED

Barth, L. G., 1938. Quantitative studies of the factors governing the rate of regeneration of Tubularia. *Biol. Bull.,* **74**: 155–177.

Barth, L. G., 1940a. The relation between oxygen consumption and rate of regeneration. *Biol. Bull.,* **78**: 366–374.

Barth, L. G., 1940b. The process of regeneration in hydroids. *Biol. Rev.,* **15**: 405–420.

Barth, L. G., 1944. The determination of the regenerating hydranth in Tubularia. *Physiol. Zool.,* **17**: 355–366.

Goldin, A., 1942a. Factors influencing regeneration and polarity determination in *Tubularia crocea. Biol. Bull.,* **82**: 243–254.

Goldin, A., 1942b. A quantitative study of the interrelationship of oxygen and hydrogen ion concentration in influencing Tubularia regeneration. *Biol. Bull.,* **82**: 340–346.

Hyman, L. H., 1926. The axial gradients in hydrozoa. VIII. Respiratory differences along the axis in Tubularia with some remarks on regeneration rate. *Biol. Bull.,* **50**: 406–426.

Miller, J. A., 1942. Some effects of covering the perisarc upon Tubularia regeneration. *Biol. Bull.,* **83**: 416–427.

Rose, S. M., and F. C. Rose, 1941. The role of a cut surface in Tubularia regeneration. *Physiol. Zool.,* **14**: 328–343.

Vol. 104, No. 2 April, 1953

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

---

# AN ANALYSIS OF THE MOLTING PROCESS IN THE FIDDLER CRAB, UCA PUGILATOR[1]

J. BRUCE GUYSELMAN[2]

*Department of Biological Sciences, Northwestern University, Evanston, Illinois*

The spectacular manner by which decapod crustaceans accomplish growth through molting has fascinated biologists for a long time. Considerable information has been accumulated during the past twenty years with respect to physiological changes which accompany molt. One of the results of these investigations has been the establishment of an hypothesis that hormones act as regulatory factors of certain processes associated with growth and molt.

Koller (1930) was the first to demonstrate a difference between normal and eyestalkless crustaceans in the inorganic content of exuviae; Koller used the shrimp, *Crangon* (*Crago*) *vulgaris.* Plankemann (1935) made similar observations for some other crustaceans. The first experimental demonstration that the sinus gland was involved in the regulation of molt was made for the crayfish, *Cambarus,* by Brown and Cunningham (1939); these investigators showed that acceleration of molt resulted from eyestalk removal, and that following eyestalk removal, the accelerated molting was retarded by sinus gland implants. Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941a) noted that eyestalk ablation in *Uca pugilator* shortened the intermolt period, but these investigators did not interpret their results as a demonstration of hormonal control of molt. A thorough discussion of crustacean hormones and their known actions is contained in a review by Brown (1952).

Alterations in contents of certain inorganic substances during the premolt and postmolt periods have been studied by Kleinholz and Bourquin (1941b) and by Guyselman (1950). Kleinholz and Bourquin determined the calcium content of exuviae from destalked *Uca* but presented no similar data for the exuviae from normal animals; however, the same investigators determined calcium values for the exuviae from destalked and normal *Palaemonetes* and found no appreciable difference between them. Guyselman found no difference between the calcium contents of exuviae of normal and destalked *Uca pugilator,* although a difference in the total ash was observed. The only other investigation pertaining to the metabolism of inorganic substances of *Uca* was that of Edwards (1950) who found that eyestalk removal effected an increase in oxygen consumption. He suggests, without adequate evidence, that the calcium content of the blood is regulated by the sinus gland and that through effecting calcium changes, the sinus gland controls oxygen consumption and other processes known to be regulated by this gland.

---

[1] This investigation is a portion of a dissertation submitted in partial fulfilment of the requirements for the Ph.D. degree in biology at Northwestern University in June, 1952. The author wishes to express his sincere appreciation to Dr. Frank A. Brown, Jr. for his advice and helpful criticism during the course of this investigation.

[2] Present address: Department of Zoology, Carleton College, Northfield, Minnesota.

The study of growth and molt in *Uca pugilator* has been approached, largely, in a qualitative manner. Abramowitz and Abramowitz (1940) demonstrated that eyestalk ablation leads to substantially increased sizes of the postmolt animals. Kleinholz and Bourquin (1941a) presented data describing weight increases associated with molting in destalked animals, but no dimensional measurements were given; the destalked animals showed a weight increase of about 30%. None of their control animals molted during the experimental period; therefore, there was provided no basis for a comparison of the normal animal with the destalked.

A number of other crustaceans have been investigated with respect to growth, molt, and mineral metabolism, and the mechanisms responsible for their regulation. Baumberger and Olmsted (1928) have discussed osmotic pressure and water changes which are associated with molt in *Pachygrapsus*. Robertson (1937, 1941) has undertaken an analysis of the inorganic composition of British shore crabs; he has studied changes associated with molting, including the absorption of water at ecdysis. Drach (1939) has made excellent studies on several European forms, dealing with the molting cycle in normal animals. Numanoi (1939), Maluf (1940), Smith (1940) and Scudamore (1947) have contributed information on various aspects of the calcium metabolism in crustaceans. Kincaid and Scheer (1952) have discussed the influence of the sinus gland on the tissue composition during the intermolt cycle of *Hemigrapsus nudus*.

## Materials and Methods

### *Animals*

The fiddler crab, *Uca pugilator,* was used for these investigations. The animals were collected during low tide in salt marshes at Chappaquoit, Massachusetts, where they occurred in great abundance. Both males and females were utilized, but the great variation in size of the large male chela rendered the males less desirable for this study. All of the animals used were collected during July and August of 1950 and 1951; wet weights and dimensions were taken only on those collected in 1951. Of 300 animals brought into the laboratory, the carapace width ranged from 1.0 to 1.9 cm., with a mean of 1.55 cm. Figure 1 shows the relationship between the cube of the carapace width and the wet weight for female animals. Conspicuous differences in coloration were noted among the animals at the time of collection and in the course of laboratory study; these differences will be discussed later.

The stock animals were kept on sea-water tables in running sea water; the floor of the table was of such a character that the water level varied over its surface from a quarter of an inch to about an inch (sufficient to cover completely an animal). For the purpose of isolating animals in sea water, No. 5 wax-coated vendor cups were punctured on two sides near their bases and arranged on the sea-water table to insure complete water coverage of a contained animal. During other experiments, including those conducted at Evanston, Illinois, animals were isolated in 4-inch finger bowls containing sea water. The only food available to the animals during the investigations was the planktonic material which was carried in the sea water. No special attempt was made to feed the animals during the course of the work. The temperature of the sea water varied between 21° and 24° C. during the investigations. Records of intensities and durations of illumination were not made; it can be stated, however, that all of the animals in the laboratory were exposed to natural and artificial light for a total period of about 20 hours daily.

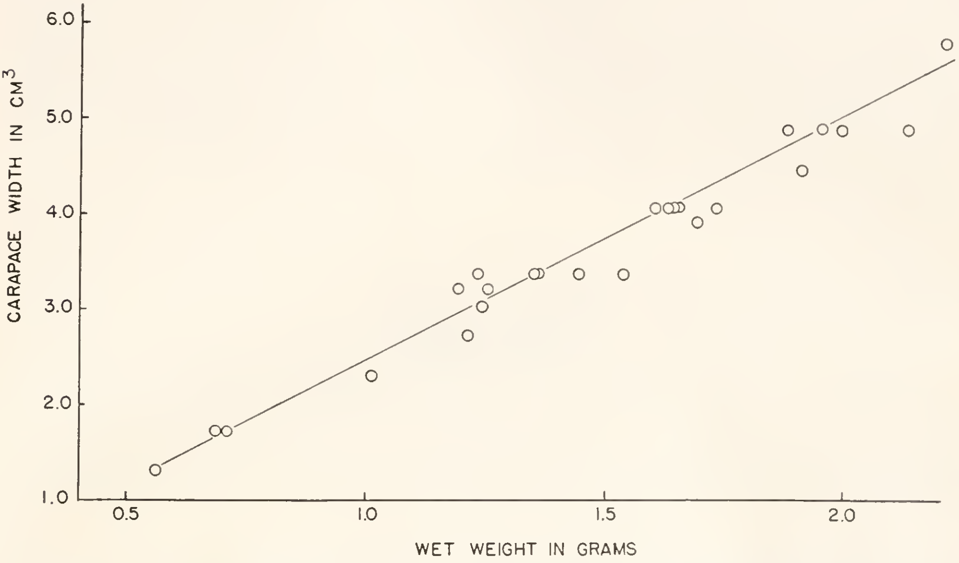


FIGURE 1. The relationship of wet weight to the cube of the carapace width in female *Uca pugilator*.

*Methods*

1. Operations

Bilateral eyestalk removal was effected by cutting off both eyestalks at their proximal ends, insuring complete removal of the sinus gland-X organ complexes contained within the stalks.

2. Weights

All weights were determined on an analytical balance.

*Wet weights* were obtained by leaving an animal for three minutes on a 4-inch filter paper in a dry finger bowl; thereafter, the animal was rolled briefly in a second piece of filter paper, placed in a porcelain crucible (ca. 25 grams) and weighed. To determine whether or not this procedure gave results which were reproducible, a single animal was weighed ten times throughout the course of two hours; the mean weight was found to be 0.9541 grams, with a standard deviation of ± 0.0045 grams.

*Dry weights* were obtained after an animal had been subjected to one or more dorsal incisions, left in a drying oven at 90° C. for 24 hours, and then left in a desiccator for at least one hour prior to weighing.

*Ash weights* were ascertained after the crucible containing a dried animal had been left in a Hoskins electric furnace at 1100–1150° C. for ten minutes and then in a desiccator for two hours. All of the desiccators contained colloidal silica gel, potassium hydroxide, and a container of concentrated sulfuric acid. This prevented any hydration of the dried or ashed animal, and reduced the possibility of recombination of the ash with atmospheric carbon dioxide. The ashing temperature was sufficient to convert, quantitatively, pure calcium carbonate to calcium oxide and carbon dioxide, and to decompose pure tricalcium-tertiary-phosphate to a complex containing 0.79 grams of ash per 1.00 gram of reagent phosphate.

### 3. Chemical analyses

*Ionic calcium* content was determined by a modification of the "Versenate" titrational method (Guyselman, 1951).

*Phosphorus* content was determined by the Fisk-Subbarow method; the measurement was made on a Coleman Junior Spectrophotometer.

*Carbon dioxide* was measured by treating a dried animal with 4 N hydrochloric acid in such a manner that the resulting gaseous expansion occurred in a closed system of adjustable volume. With this apparatus, the volume of gas generated was readily measured; the error was found to be less than 2% when 0.090 grams of pure calcium carbonate were used.

### *Terminology applied to the molting process*

Other investigators have employed various terminologies to describe the various phases and events associated with the molting cycle. The following classification of phases will be used for this work:

1. Intermolt—that period, if any, during which there is a maintenance of a steady-state of the hardened cuticle. It is extremely variable in duration under certain conditions, and may even be lacking in zooecial stages.
2. Premolt—that period during which certain processes lead to epidermal separation.
3. Molt—a time of epidermal separation from the old cuticle, of the formation of a new cuticle, and of the subsequent shedding (ecdysis) of the old cuticle (exuvia).
4. Postmolt—a period of cuticular expansion and hardening, gradually leading either to the intermolt or premolt condition.

## Experiments and Results

### A. *Premolt*

### 1. Color changes

It was stated earlier that differences in coloration were observed in freshly collected animals. Since it had been reported that certain changes in pigmentation were a visible indication of active processes leading to ecdysis in some of the

Brachyura (Drach, 1939), the differences in coloration in *U. pugilator* were studied with reference to such a possible relationship.

Cursory observations made during the summer of 1950 on animals retained for some time in the laboratory indicated that the gross pigmentary pattern of an animal in which ecdysis was to occur in two or three days differed from that of individuals ordinarily collected in the field. The former animals were observed to be blue-gray in color, in contrast to the specific patterns of brown, light brown, and purple possessed by the latter and giving the species the colloquial name, "calico backs." However, there seemed to be no indication of any progressive gross color changes preceding the adoption of the blue-gray color which could be regarded as an indication of approaching ecdysis.

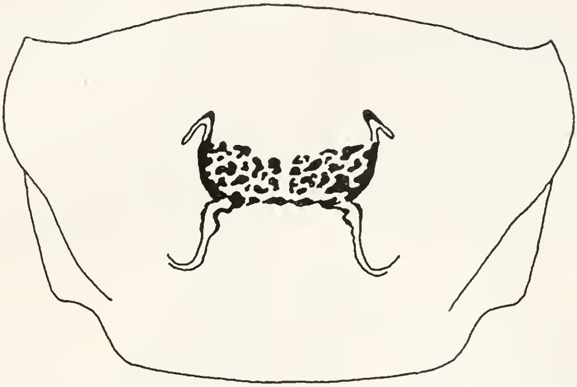


FIGURE 2. Dorsal aspect of the carapace of *Uca pugilator* showing the dorsal crest. The appendages have been removed.

In a group of animals collected on July 29, 1951, the type of coloration in a small region of the carapace served as a means of segregating the animals into three separate groups. Figure 2 is a line drawing of the carapace, showing this distinctive region which will be referred to subsequently as the "dorsal crest." The three groups showed the following differences in the dorsal crest:

Group 1: white patterns on a brown field (Fig. 3a)
Group 2: pale orange and white patterns on a brown field (Fig. 3b)
Group 3: indistinct gray and pale orange patterns on a gray-brown field (Fig. 3c)

During the course of daily observations, a fourth pattern of coloration appeared within animals of group 3. This fourth group had the following characteristics:

Group 4: yellow-orange patterns on a light gray-brown field (Fig. 3d)

Similarly, animals now in group 4 were the source of a fifth pattern which was observed:

Group 5: light yellow patterns on a light blue-gray field (Fig. 3e). Animals in this condition had a gross blue-gray color and were almost identical with those observed in 1950 which were within a few days of ecdysis.

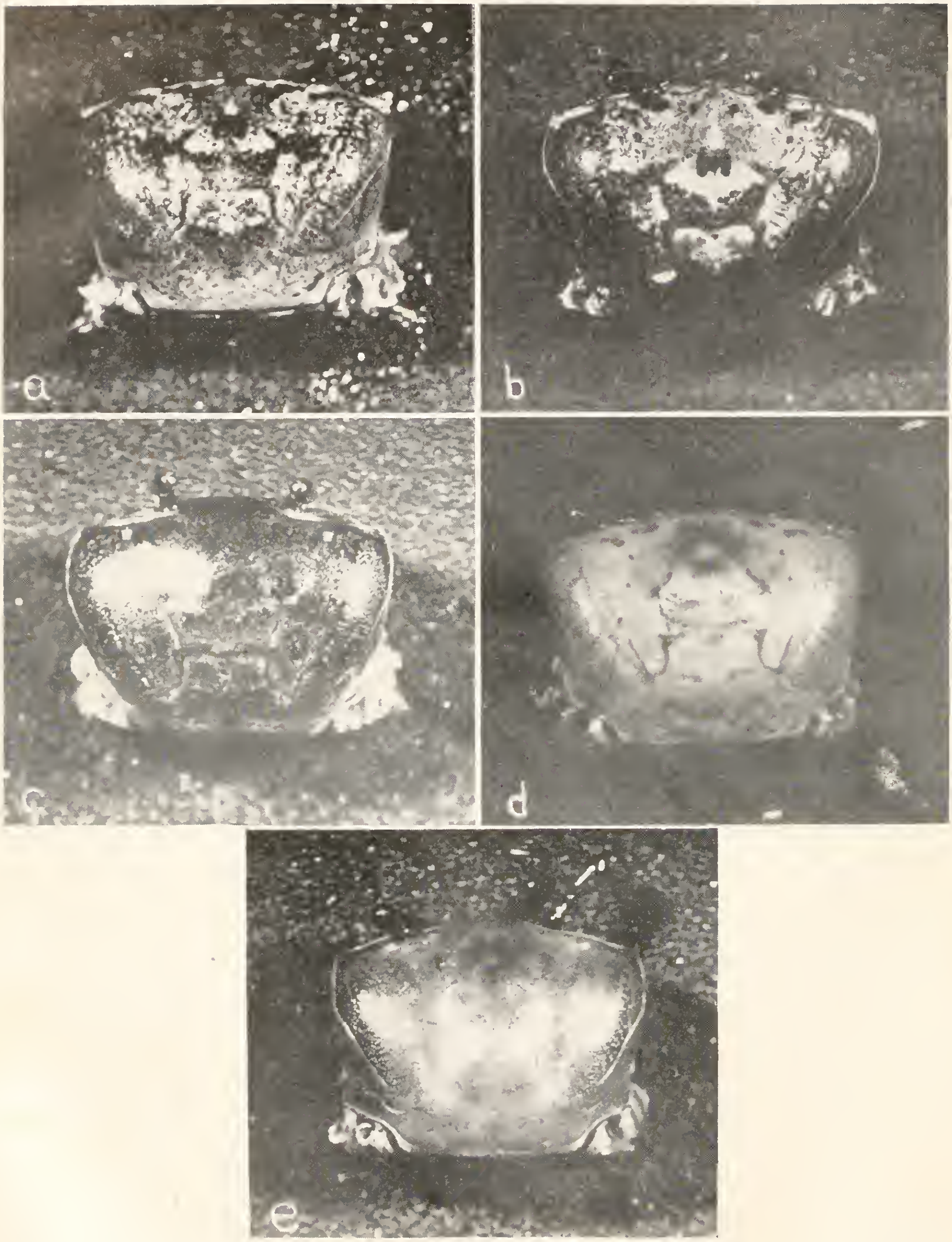


FIGURE 3. Photographs of normal *Uca pugilator* in each of the five stages: (a), stage one; (b), stage two; (c), stage three; (d), stage four; (e), stage five.

Table I shows changes in the number and percentages of animals contained within each of the five groups over a six-day period. It appeared obvious from these data that a progressive change in coloration was occurring in a direction from group 1 to group 5, but evidence was incomplete in that no single animal was seen to make a complete transition from 1 to 5.

Figure 4 shows the changes in group status of a single animal initially in group 1 over the course of 23 days. That this transition occurred in the direction from 1 to 5 was now established beyond doubt. Consequently, the term "stage" was now adopted in place of "group." Furthermore, the length of time that an animal remained in each of stages 2 through 5, under these laboratory conditions, was established. The duration of stage 1 was not determined but the coloration characteristics of an animal immediately following ecdysis are those of a stage 1 animal, as is shown by Figure 5. During a period of a week or two after ecdysis, the maximum time that such animals were observed, no visible changes in coloration occurred. There is nothing to suggest that any coloration change occurs between ecdysis and the premolt period. Therefore, stage 1 is presumed to extend throughout the intermolt period.

TABLE I

*Inter-group transition in normal U. pugilator*

| Date | Percentage of animals in each group | | | | | Number of animals |
|---|---|---|---|---|---|---|
| | Group: | | | | | |
| | 1 | 2 | 3 | 4 | 5 | |
| 7-29-51 | 24% | 27% | 49% | 0% | 0% | 56 |
| 7-31-51 | 24 | 26 | 50 | 0 | 0 | 56 |
| 8- 1-51 | 24 | 25 | 46 | 5 | 0 | 56 |
| 8- 2-51 | 23 | 24 | 24 | 23 | 6 | 52 |
| 8- 3-51 | 15 | 16 | 27 | 26 | 16 | 52 |
| 8- 4-51 | 5 | 18 | 28 | 36 | 13* | 50 |

* Of the seven animals in group five, two underwent ecdysis, three died, and two were sacrificed for inorganic analyses.

No attempt was made to interpret these changes in coloration at the histological level; however, it can be shown that this transition is correlated with the process of formation of the new cuticle and its separation from the old exoskeleton, and probably also with the partial resorption of the old cuticle. Figure 5a shows a stage 5 animal with a portion of the carapace removed; the new cuticle (with stage 1 characteristics) has separated completely from the old cuticle. Similar examinations were made on animals in other stages; only in 3, 4 and 5 was cuticular separation clearly evident. Considerable variation, however, existed in stage 3 animals with regard to the completeness of cuticular separation; in some cases it was still attached, at least in part, to the carapace while in others it had separated.

During the remainder of the summer and fall of 1951 three additional series of animals were isolated and examined daily for progressive stage changes. The re-

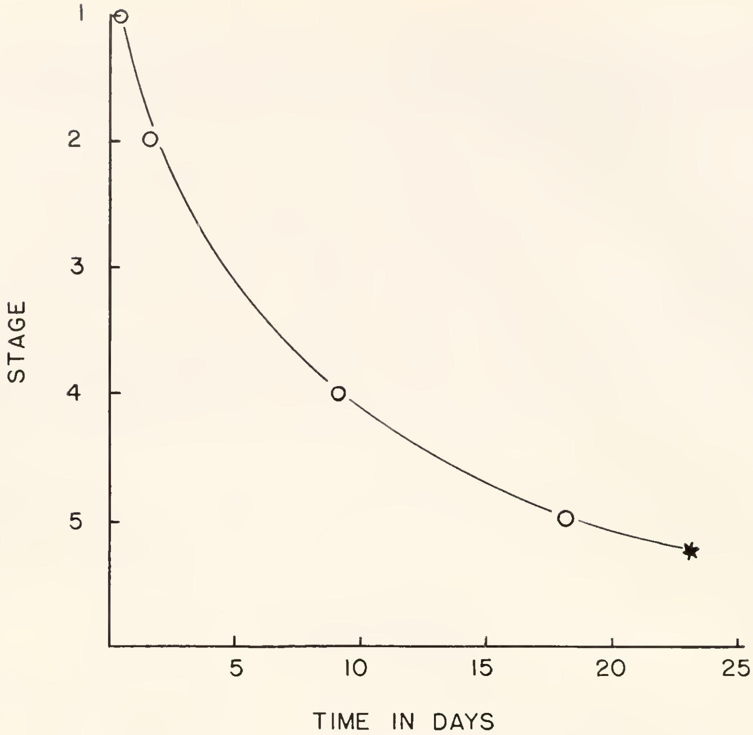


FIGURE 4. Interstage transition for a single, female *Uca pugilator* from stage one until ecdysis; *ecdysis.

sults of these investigations are given in Table II. It appears evident from these results that interstage transition does not occur at the same rate at all times of the year. This is further substantiated by the decreased incidence of ecdysis in series B and C animals.

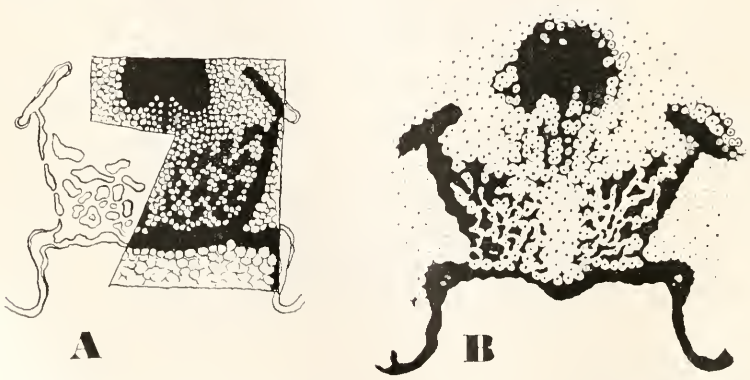


FIGURE 5. Carapace coloration of *Uca pugilator* in stage five (a) showing portion of new cuticle, and carapace coloration in the postmolt condition (b).

During these investigations of the changes in coloration of the carapace in normal animals prior to ecdysis, an attempt was made to determine whether or not a similar series of changes took place in destalked animals. Observations made during the summer of 1950 on destalked animals seemed to indicate that there was little or no difference in gross coloration between animals that had been destalked for a day or two and animals which were within a few hours of ecdysis. Furthermore, it had been shown that one of the effects of bilateral eyestalk removal on *U. pugilator* was a paling of the carapace (Abramowitz and Abramowitz, 1940). Daily observations on a series of animals destalked July 28, 1951, showed no indication of transitional color changes; in all instances these animals assumed a light gray-tan color and retained to some extent their previous dorsal crest coloration until the time of ecdysis.

TABLE II

*Inter-stage transitions at different times of the year during five-day periods. Percentages represent the distribution at the beginning and at the end of five-day periods*

| Stage | Group A<br>7-31-51<br>to<br>8-4-51 | | Group B<br>8-11-51<br>to<br>8-15-51 | | Group C<br>10-22-51<br>to<br>10-27-51 | |
|---|---|---|---|---|---|---|
| 1 | 24% | 5% | 35% | 13% | 24% | 25% |
| 2 | 26 | 18 | 30 | 27 | 64 | 66 |
| 3 | 50 | 28 | 27 | 21 | 8 | 6 |
| 4 | 0 | 36 | 6 | 3 | 0 | 0 |
| 5 | 0 | 13 | 2 | 0 | 0 | 0 |
| Total no. of animals observed | 100 | 64 | 56 | 50 | 30 | 30 |
| Total no. of animals that molted | | 2 | | 0 | | 0 |

2. Inorganic constituents of normal animals

In view of the fact that visible alterations in coloration occurred in normal crabs antecedent to ecdysis in such a regular manner that the approximate time of the subsequent ecdysis could be predicted, a method was provided by which chemical analyses of animals could be made at determinable periods prior to ecdysis. It will be recalled that the staging by coloration had not been perfected in time for the studies made in 1950; therefore, the analyses made at that time could be correlated only with three phases: the first including stage 1 and 2; the second, 3; and the last, 4 and 5. In the analyses to be described in this section, stage 1 animals were restricted to those which, as far as could be determined, had completed their postmolt hardening of the exoskeleton.

### *Calcium*

It has been reported that calcium constitutes 16.3% of the dry weight of a freshly killed *U. pugilator* (Kleinholz and Bourquin, 1941b). This value is based on the average obtained with five animals. Table III contains figures for the dry weight, ash

TABLE III

*Inorganic constituents of normal U. pugilator*

| Animal no. | Sex | Width (cm.) | Stage | Dry weight (gms.) | Ash weight (gms.) | Calcium (gms.) | Calcium as % of dry weight |
|---|---|---|---|---|---|---|---|
| C99 | F | 1.6 | 1 | 0.7909 | 0.1627 | 0.072 | 9.1 |
| C84 | F | 1.6 | 3 | 0.4903 | 0.1420 | 0.058 | 11.9 |
| B16 | F | 1.6 | 3 | 0.5283 | 0.1929 | 0.048 | 9.0 |
| C42 | F | 1.65 | 2 | 0.6471 | 0.1703 | 0.078 | 12.0 |
| C101 | M | 1.5 | 5 | 0.7754 | 0.2600 | 0.084 | 10.8 |
| C102 | M | 1.65 | 1 | 0.7628 | 0.3224 | 0.106 | 13.8 |
| C103 | M | 1.7 | 3 | 0.8124 | 0.3048 | 0.114 | 14.0 |
| C31 | F | 1.65 | 1 | 0.6010 | 0.1586 | 0.072 | 11.9 |
| D29 | M | 1.8 | 2 | 0.5625 | — | 0.078 | 15.4 |
| C111 | M | 1.0 | 2 | 0.1415 | — | 0.020 | 14.2 |
| 10 | M | 2.2 | 1–2 | 0.7394 | 0.2704 | 0.084 | 11.4 |
| 55 | M | — | 1–2 | 0.6737 | 0.2150 | 0.128 | 19.0 |
| 56 | M | — | 1–2 | 0.6483 | 0.2110 | 0.101 | 15.4 |
| 57 | M | — | 1–2 | 0.8493 | 0.2662 | 0.139 | 16.3 |
| 8 | M | — | 4–5 | 1.1359 | 0.4029 | 0.188 | 16.5 |
| 58 | M | — | 1–2 | 0.6518 | 0.2358 | 0.096 | 14.7 |
| 59 | M | — | 1–2 | 0.6975 | 0.2290 | 0.122 | 17.5 |
| 43a | M | — | 3 | 0.7052 | 0.2382 | 0.140 | 19.8 |
| C57 | M | — | 1–2 | 0.7569 | 0.2515 | 0.124 | 16.3 |
| 6 | M | — | 4–5 | 0.8175 | 0.3058 | 0.176 | 20.3 |

weight, calcium, and calcium as the percentage of dry weight for 20 animals. It will be seen that the amount of calcium ranges from 9.0% to 20.3% of the dry weight, with an average of 14.4% and a standard deviation of ± 3.4%. While 9.0% is the minimal value for an animal in stage 1, a value of 10.8% is found for an animal which appears to be within a day or two of ecdysis. From these data it would seem that there is no correlation between the stage of an animal and the amount of calcium it contains. A value of 9.0% would seem to be the minimal amount occurring in a fully hardened animal.

The most abundant forms of inorganic calcium in decapod crustaceans are carbonate and phosphate. Table IV shows the proportions in which these salts occurred in four completely hardened animals. Calcium carbonate constituted about 27% of the dry weight while calcium phosphate comprised about 9%. The ratio

TABLE IV

*Carbonate and phosphate contents of normal U. pugilator*

| Animal | Sex | Width (cm.) | Stage | Dry weight (gms.) | Calcium carbonate | | Calcium phosphate | |
|---|---|---|---|---|---|---|---|---|
| | | | | | grams | % of dry weight | grams | % of dry weight |
| C112 | F | 1.2 | 3 | 0.191 | 0.055 | 28.8 | 0.022 | 11.6 |
| C111 | M | 1.0 | 2 | 0.141 | 0.033 | 23.5 | 0.010 | 7.2 |
| D29 | M | 1.8 | 2 | 0.562 | 0.172 | 30.5 | 0.049 | 8.8 |
| D31 | F | 1.65 | 1 | 0.560 | 0.140 | 25.0 | — | — |

of the carbonate to the phosphate in animals under these conditions appears, therefore, to be approximately 3 : 1. Data are not available for all of the five stages in the molting cycle with respect to carbonate and phosphate contents. However, as will be evident later, studies of ashed animals give data which permit one to determine whether any significant alteration in this 3 : 1 ratio has occurred. These later data were obtained from animals representing all five stages.

### *Ash*

At the temperature employed for the ashing of animals, all organic matter was destroyed, calcium carbonate was quantitatively converted to calcium oxide, and calcium phosphate was decomposed to a mixture of its oxides. An average value of 32.7% was obtained for 17 animals in all of the stages; values ranged from 20.6% to 42.2% for animals in stage 1, with a mean of 29.7%. Animals in stages 2 through 5 gave values of from 29.0% to 37.4% with a mean of 33.9% and a standard deviation of ± 2.6%. The greatest variation is found in stage 1 animals, all of which, however, appeared to have completely hardened exoskeletons.

### *Water*

The water content of animals in stages 1 through 5 was found to be 67.9% with a standard deviation of ± 4.7%, values ranging from 57.1% to 74.0% for 13 animals. No significant variation in water content of hard-shelled animals was observed through the five stages. The variation in water content of stage 1 animals during the period of the hardening of the exoskeleton will be treated later. It should be mentioned that the average value of 67.9%, found in these analyses, was obtained with animals that had been kept in running sea water. For a period of ten days, relatively low weights were obtained between 7 P.M. and 8 P.M., while higher weights were found during the morning hours. Figure 6 contains values for the wet weights of an animal during the course of three days prior to ecdysis. It will be seen that during the last two days prior to ecdysis the fluctuations appear arrhythmical, in contrast to a diurnally rhythmic tendency which had been noted during the first seven days of observation. Furthermore, the amplitude of the fluctuations decreases as the animal approaches ecdysis. Immediately after ecdysis the minimal daily weight was obtained at about 6 P.M., while the maximal weight was obtained at about 6 A.M. This aspect of postmolt weight changes will be discussed in a later section. Animals kept in distilled water for 24 hours showed arrhythmic fluctuations and the amplitude of the fluctuation was considerably reduced as compared to the changes in the animal shown in Figure 6. No weight fluctuations of a rhythmic character were observed in animals kept in finger bowls of sea water in the experiments performed in Evanston, Illinois in October and November. The fluctuations observed were, however, essentially of the same amplitude as those reported for animals at the Marine Biological Laboratory during the summer.

## 3. Inorganic constituents of destalked animals

### *Calcium*

No appreciable difference could be detected between the calcium contents of normal and destalked animals. For a group of animals which had been destalked for

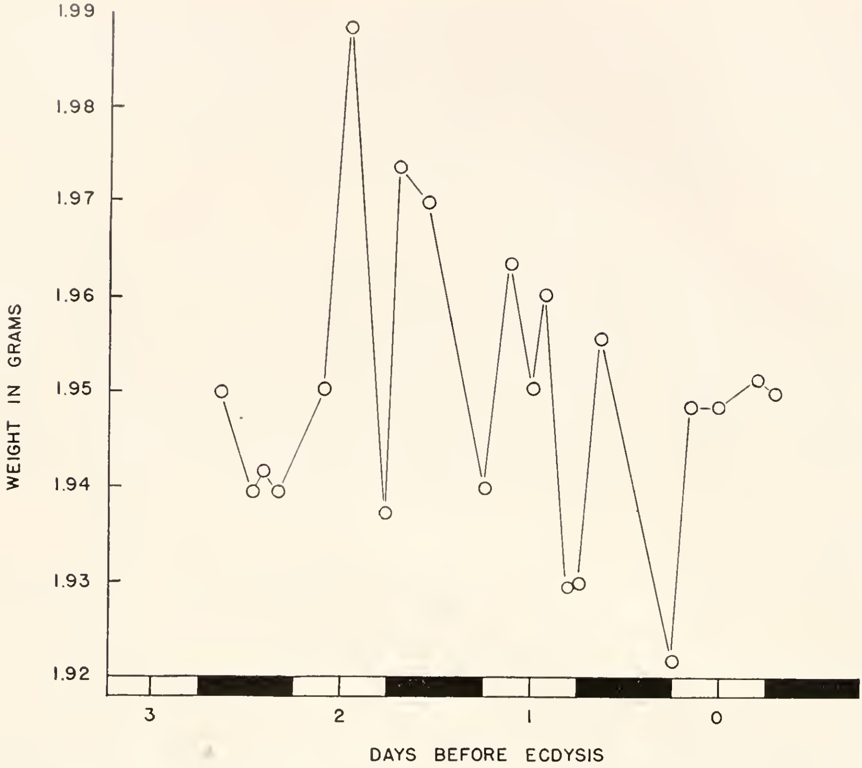


FIGURE 6. Weight changes in single, normal, female *Uca pugilator* before ecdysis. The black bar indicates a period from 6 P.M. to 6 A.M.

three, five, seven, and fourteen days, the mean percentage of calcium was found to be 16.4% with a standard deviation of ± 2.1%, values ranging from 12.9% to 18.7%. These values are well within the range found for normal animals. While calcium is not stored in the hepatopancreas of *U. pugilator* to any appreciable extent (less than 0.5% of the total dry weight in normal animals), one of the effects of bilateral eyestalk removal has been shown to be the complete depletion of calcium from the hepatopancreas within five days after the operation (Guyselman, 1950).

### *Ash*

The ash, expressed as percentage of dry weight, was found to be 36.1% with a standard deviation of ±4.7%, values ranging from 30.6% to 45.5%. Again, the average value is not significantly different from that found for normal animals. That certain changes in the inorganic composition do occur following destalking will be shown by a comparison of the exuviae of destalked animals, cast at various times after destalking, with those of normal animals.

### *Water*

The water contents of destalked animals, prior to ecdysis, can be treated only in an indirect manner. Wet weights were determined for five destalked animals during periods of six to ten days. The results are given in Table V. It does not appear likely from these data that any significant changes occurred in the water contents during this period. There may be a slight increase in weight for the first few days, but after one week the weights are essentially the same as the initial weights.

Three destalked female animals, weighed at the end of seven, eight, and twelve days after destalking, showed that there was no increase in wet weight when compared to normal animals of the same carapace width. One of these animals, 1.8 cm. in carapace width, weighed 2.06 grams on the seventh day after destalking; another, having the same carapace width, weighed 2.05 grams on the twelfth day after destalking. The third animal, 1.9 cm. in width, weighed 2.64 grams eight days after destalking. It will be seen, by inspection of Figure 1, that these values are well within the range of weight variations that are found in normal animals.

TABLE V

*Wet weights (in grams) of destalked U. pugilator*

| No. of days after destalking | Animal number | | | | |
|---|---|---|---|---|---|
| | D4 | D5 | D6 | D7 | D18 |
| 1 | 1.682 | 1.588 | 1.238 | 1.848 | 0.704 |
| 2 | 1.703 | 1.617 | 1.242 | 1.872 | 0.703 |
| 3 | 1.739 | — | — | 1.904 | — |
| 4 | 1.726 | 1.636 | 1.260 | 1.921 | 0.708 |
| 5 | 1.685 | — | 1.244 | 1.890 | — |
| 6 | 1.694 | — | — | 1.878 | — |
| 7 | — | 1.597 | 1.244 | 1.898 | 0.723 |
| 8 | — | — | 1.252 | 1.924 | — |
| 9 | — | — | 1.242 | 1.892 | — |
| 10 | — | — | — | 1.884 | — |

B. *Molt*

One of the processes which characterizes the initial phase of molt is the separation of the epidermis from the cuticle. It will be remembered that this epidermal separation was first noticed in animals which were in stage 3. The period from stage 3 to the termination of molt was from 15 to 20 days in those animals which were examined during the early part of August. The duration of stage 3 appeared to be about 6 days at this time. Therefore, assuming that molting activity of the epidermis commences during the latter part of stage 3, an animal would be engaged for about 10 days in visible aspects of the molting process, a process which is terminated by the abrupt shedding of the old exoskeleton.

Bilateral eyestalk removal, thereby eliminating both sinus gland-X organ complexes, will induce molt in *U. pugilator* (Abramowitz and Abramowitz, 1940). Table VI contains data for a group of animals which were destalked on July 28, 1951; all the animals were staged before destalking. These results, despite the small number of animals used and the variability observed, nevertheless suggest that there is an inverse correlation between the stage number of an animal at the time of destalking and the time elapsing between destalking and ecdysis. For animals in stages 1 and 2, 18 days elapsed; for stage 3, 14 days elapsed; and for stage 4, 10 days elapsed. It is especially noticeable that stage 3 animals show a dichotomy with respect to the interval between destalking and ecdysis, being of the order of either 6 to 8 days, or 15 to 25 days.

The actual process of shedding the old exoskeleton is relatively rapid; normal animals were observed to complete the casting process in about 15 minutes. Such animals showed little motor activity for several hours prior to ecdysis; occasionally these animals and ones in stage 4 were observed to exhibit non-locomotor movements of the walking appendages. This activity was never observed in animals in stages 1, 2, and 3.

TABLE VI

*Relationship of color-stage in destalked U. pugilator to interval between destalking and ecdysis*

| Animal no. | Sex | Carapace width (cm.) | Stage at time of destalking | Number of days after destalking that ecdysis occurred |
|---|---|---|---|---|
| 11 | F | 1.5 | 1 | 15 |
| 27 | F | 1.6 | 1 | 18 |
| 15 | F | 1.7 | 2 | 19 |
| 14 | F | 1.7 | 2 | 21 |
| 22 | F | 1.6 | 2 | 17 |
| 8 | F | 1.5 | 2 | 14 |
| 17 | M | 1.9 | 2 | 14 |
| 6 | M | 1.6 | 2 | 27 |
| 35 | M | 1.5 | 3 | 18 |
| 45 | M | 1.6 | 3 | 14 |
| 41 | M | 1.7 | 3 | 27 |
| 5 | M | 1.7 | 3 | 15 |
| 13 | F | 1.3 | 3 | 8 |
| 9 | F | 1.5 | 3 | 6 |
| 40 | F | 1.5 | 3 | 12 |
| 47 | F | 1.6 | 3 | 14 |
| 10 | F | 1.6 | 3 | 18 |
| 37 | F | 1.6 | 3 | 18 |
| 30 | F | 1.7 | 3 | 7 |
| 46 | F | 1.7 | 3 | 13 |
| 25 | F | 1.7 | 4 | 15 |
| 44 | F | 1.4 | 4 | 5 |

The first indication of ecdysis was a separation at the cephalothoracico-abdominal junction. This was followed by a splitting along the lateral margins of the exoskeleton; as the posterior and lateral margins of the carapace split, the animal underwent active muscular movements, gradually withdrawing itself from the exuvia.

Many of the destalked animals did not free themselves completely from the exuviae, certain anterior portions of the alimentary tract remaining attached. This apparent failure of destalked animals to emerge successfully was also observed by Abramowitz and Abramowitz (1940).

1. Uptake of water

Table VII gives the weights of three animals prior to ecdysis, the weights within 15 minutes after emergence, the weights of the cast exuviae, and the increase in weight due to water absorption. In addition, the table includes the weights of the freshly molted animals and the water absorbed, expressed as percentages of the weights immediately prior to ecdysis. It will be seen that the amount of water absorbed at ecdysis is inversely proportional to both weights and carapace widths of the animals before ecdysis. It was found that the product of the cube of the carapace width and the weight of the water absorbed (expressed as the percentage of the weight prior to ecdysis) was relatively constant; for the animals indicated in Table VII, a mean product of 170.0 with a standard deviation of ± 4.7 was obtained. This indicates that the increase in water content at ecdysis appears to be a function of the volume of an animal, with the smaller animals absorbing a higher percentage than the larger ones.

TABLE VII

*Changes in wet weights associated with ecdysis in normal, female U. pugilator*

| Animal no. | Width (cm.) | W1 | E1 | W2 | W3 | E1 as % of W1 | W2 as % of W1 | W3 as % of W1 |
|---|---|---|---|---|---|---|---|---|
| | | (grams) | | | | | | |
| C11 | 1.7 | 1.960 | 0.899 | 1.740 | 0.679 | 45.8 | 89.0 | 34.6 |
| 51F | 1.6 | 1.619 | 0.819 | 1.458 | 0.658 | 50.6 | 91.8 | 40.6 |
| 5F | 1.5 | 1.540 | 0.868 | 1.494 | 0.822 | 56.4 | 96.8 | 53.4 |

W1 = wet weight before ecdysis
E1 = wet weight of exuvia
W2 = wet weight after ecdysis
W3 = W2 − (W1 − E1) = weight of water absorbed at ecdysis

It was demonstrated earlier that the water content of animals before ecdysis is about 68%. The water content of an animal 15 minutes after ecdysis is approximately 84%, values of 84.5% and 84.1% having been obtained with two animals. Although this value of 84% has been derived from direct determinations using only two animals, it is readily possible to calculate the water contents of the three animals described in Table VII immediately following ecdysis. When this is done, the three values are 82.3%, 89.6%, and 83.1%. The average for these values is 85.0%, which is in close agreement with those obtained by direct determinations.

Sufficient data are lacking for destalked animals with respect to water uptake at ecdysis. However, exploratory research seems to indicate that the increase in weight accompanying ecdysis is approximately the same as that found for normal animals. Kleinholz and Bourquin (1941a) determined the percentage weight increases in destalked *U. pugilator* following ecdysis; however, initial and final weights were not given. They state that of six animals in the 1.6 to 2.0 gram size-class, the average percentage gain in weight after molting is 29.83 ± 7.20%. This value would indicate that, under certain conditions, destalked animals may absorb even less water than normal animals at ecdysis.

## 2. Weight changes

Figures 7a and 7b show the weight changes in two postmolt animals during a period of five days. It will be seen that at the end of this period the animals have not regained the weight they had prior to ecdysis. None of the animals was weighed at a later time than ten days after ecdysis.

An inspection of the plotted weights in Figures 7a and 7b suggests, again, a rhythmical variation, with the greatest weights occurring at about 6 A.M., and the

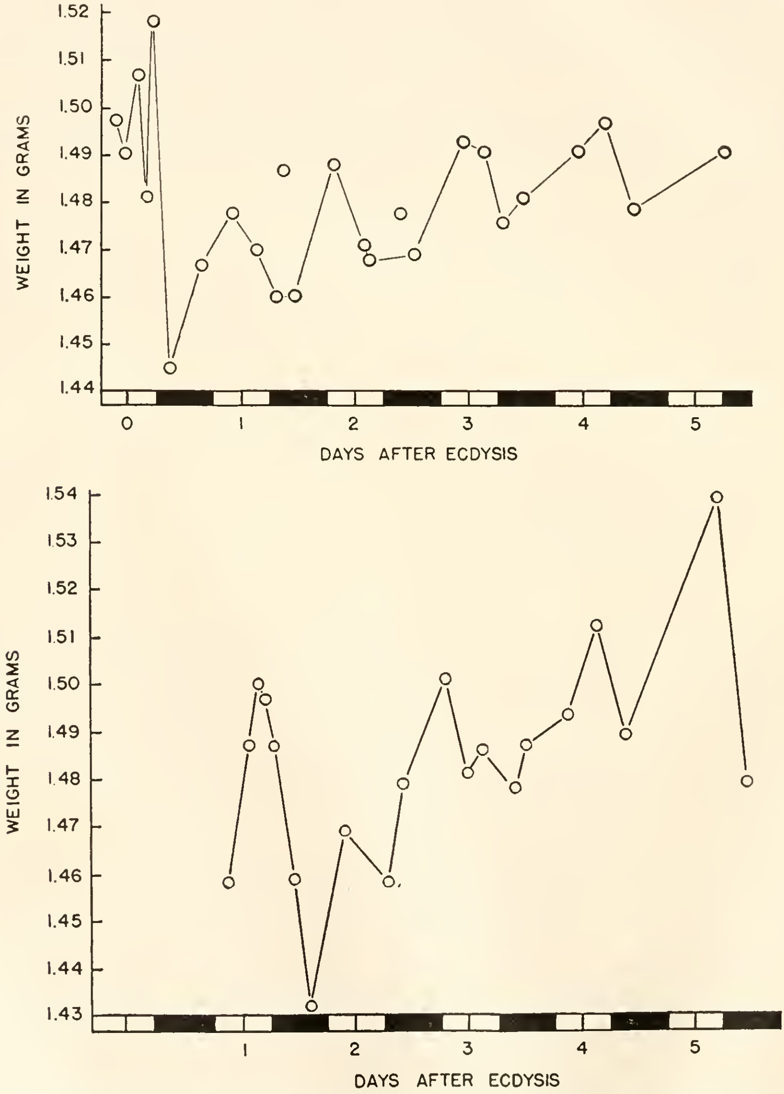


FIGURE 7. Weight changes of two, normal, female *Uca pugilator* (a and b) following ecdysis. The black bar indicates a period from 6 P.M. to 6 A.M.

TABLE VIII

*Changes in carapace width in normal and destalked U. pugilator following ecdysis*

| Animal no. | Condition | Width before ecdysis (cm.) | Days after ecdysis that new dimensions were taken | Width after ecdysis (cm.) | Percentage increase in width |
|---|---|---|---|---|---|
| 40 | Destalked | 1.5 | 0 | 1.6 | 6.7 |
| 14 | Destalked | 1.7 | 2 | 1.8 | 5.9 |
| 41 | Destalked | 1.7 | 3 | 1.8 | 5.9 |
| 35 | Destalked | 1.5 | 5 | 1.6 | 6.7 |
| 13 | Destalked | 1.3 | 7 | 1.35 | 3.8 |
| 49 | Destalked | 1.6 | 8 | 1.8 | 12.5 |
| 25 | Destalked | 1.7 | 8 | 1.9 | 11.8 |
| 15 | Destalked | 1.5 | 18 | 1.8 | 20.0 |
| A26 | Normal | 1.6 | 1 | 1.6 | 0 |
| C105 | Normal | 1.3 | 2 | 1.3 | 0 |
| 51F | Normal | 1.6 | 5 | 1.6 | 0 |
| 5F | Normal | 1.5 | 5 | 1.5 | 0 |
| 124 | Normal | 1.3 | 7 | 1.3 | 0 |
| C11 | Normal | 1.7 | 7 | 1.7 | 0 |

smallest at about 6 P.M. The rhythmical consistency of the fluctuations in weights which was obtained for these animals suggests strongly that some factor other than error inherent in the technique in weighing is responsible for this orderly fluctuation.

### 3. Dimensional changes

Of primary interest is the fact that none of the three normal animals, discussed above in Table VII, showed any change in carapace width during the ten-day period following ecdysis; all three retained their pre-ecdysis dimensions. Three additional animals, the weights of which were not obtained (hence they received relatively little handling), similarly failed to show an increase in width.

TABLE IX

*Inorganic constituents of normal and destalked, postmolt U. pugilator*

| Animal no. | Condition and days postmolt | Dry weight (gms.) | Ash | | Calcium | |
|---|---|---|---|---|---|---|
| | | | (gms.) | % of dry weight | (gms.) | % of dry weight |
| 3E | N 2 hrs. | .1478 | .0389 | 26.4 | .010 | 6.7 |
| C105 | N 2 days | .1329 | .0395 | 29.7 | — | — |
| 26 | N 5 days | .3725 | .0960 | 26.5 | .019 | 5.1 |
| 124 | N 7 days | .1811 | .0564 | 31.0 | .017 | 9.1 |
| 39 | D 1 day | .2678 | .1042 | 39.3 | .010 | 3.7 |
| 20b | D 4 days | .3692 | .1385 | 37.4 | .041 | 11.0 |
| 13 | D 7 days | .1474 | .0502 | 35.0 | .010 | 6.8 |
| 49 | D 7 days | .3015 | .1098 | 36.5 | .031 | 10.2 |
| 35b | D 12 days | .2552 | .0528 | 20.7 | .013 | 5.2 |
| 31 | D 17 days | .3417 | .1034 | 30.2 | .021 | 6.2 |
| 15 | D 18 days | .3406 | .1213 | 32.5 | — | — |

Destalked animals, on the contrary, did increase in carapace width and exhibited this increase soon after ecdysis. Table VIII contains values for carapace widths of destalked animals before and after ecdysis, and the percentage increase over the width before ecdysis. For purposes of comparison similar data are presented for the six normal animals. It will be seen, therefore, that at least for a postmolt period of seven days, destalked animals present a striking contrast to normal animals.

### 4. Hardening

Table IX contains data for the inorganic composition of a group of postmolt animals, including both normal and destalked. One of the most notable differences between the two is the relatively high ash content of destalked forms. It now becomes of interest to examine the composition of the cast exuviae of destalked and normal animals. This information is given in Table X. Indicated are the dry weights, ash weights, calcium, and the ash and calcium as percentages of the dry weights. It will be seen that animals which undergo ecdysis within five or six days after destalking shed an exuvia which contains about 53% ash; if ecdysis occurs later than six days after destalking, the ash content is about 47%. This value of 47% is found for exuviae of destalked animals which are cast between six and 27 days after destalking. In contrast, normal animals shed an exuvia which contains about 61% ash. The percentage of calcium, however, is about 27% for exuviae from both normal and destalked animals. That the calcium content of exuviae from destalked animals is about 27% was reported by Kleinholz and Bourquin (1941b). It will be seen by an inspection of Tables IX and X that the extent of the reduction in ash content in exuviae of destalked animals, in comparison with normal, corresponds closely with the proportionally higher ash content which is observed in destalked postmolt animals, a value of approximately 10% having been obtained in both cases.

TABLE X

*Relationship in destalked animals of the ash and calcium contents of the exuviae to the interval between destalking and ecdysis. Exuviae of two normal animals are included for comparison*

| Exuvia no. | Days after destalking that animal underwent ecdysis | Dry weight (gms.) | Ash | | Calcium | |
|---|---|---|---|---|---|---|
| | | | (gms.) | % of dry weight | (gms.) | % of dry weight |
| 3E | 2 hours | .2430 | .1233 | 50.0 | .052 | 21.5 |
| A18 | 5 days | .2228 | .1409 | 63.5 | — | — |
| 44 | 6 days | .1919 | .0919 | 48.0 | — | — |
| 9 | 6 days | .2056 | .1084 | 53.0 | .058 | 28.2 |
| A19 | 7 days | .1570 | .0704 | 44.0 | — | — |
| 13 | 8 days | .1704 | .0830 | 49.0 | .047 | 27.8 |
| 30 | 10 days | .2698 | .1296 | 48.0 | .076 | 28.6 |
| A26 | 10 days | .2530 | — | — | .073 | 29.0 |
| 6 | 27 days | .3279 | .1571 | 47.0 | — | — |
| 41 | 27 days | .3939 | .1919 | 48.0 | — | — |
| | Normal animals | | | | | |
| 55 | | .4396 | .2584 | 59.0 | .122 | 27.7 |
| 5F | | .2324 | .1444 | 64.0 | — | — |

## Discussion

One of the effects of bilateral eyestalk removal in *Uca pugilator* has been shown through these investigations to be a reduction of the non-calcium inorganic content of the exoskeleton, and the apparent transfer of this material into the soft tissues of the animal (*cf.,* Tables IX and X). Since the calcium content of the exoskeleton is not appreciably altered by eyestalk ablation, it would appear that the sinus gland-X organ complex exerts a specific influence not on calcium metabolism *per se,* but rather on processes which control anionic metabolism. Such a mode of action would affect the calcium level in an indirect manner. In all probability a similar situation occurred in Koller's (1930) investigations of *Crago;* he reported that the acid-soluble mineral content of cast exuviae from destalked animals was less than that found in cast exuviae of normal animals. Furthermore, the results of Kleinholz and Bourquin (1941b), indicating no difference between exuviae of normal and destalked *Palaemonetes* with respect to the calcium contents, may be attributable to a similar mode of action.

Table XI

*Calculations in terms of various forms of calcium for normal and destalked U. pugilator*

| Animal no. | Number of days after ecdysis | Condition | Calcium<br>A calculated as 100% calcium oxide | <br>B calculated as 100% calcium phosphate | A as % of ash | B as % of ash |
|---|---|---|---|---|---|---|
| 39 | 1 | Destalked | .014 | .020 | 13.4 | 19.2 |
| 20b | 4 | Destalked | .057 | .084 | 41.0 | 60.8 |
| 13 | 7 | Destalked | .014 | .020 | 28.0 | 40.0 |
| 49 | 7 | Destalked | .043 | .063 | 39.4 | 57.6 |
| 35b | 12 | Destalked | .018 | .026 | 34.1 | 49.2 |
| 31 | 17 | Destalked | .029 | .043 | 28.0 | 41.6 |
| 3E | 0 | Normal | .014 | .020 | 36.0 | 51.2 |
| 26 | 5 | Normal | .026 | .039 | 27.0 | 40.7 |
| 124 | 7 | Normal | .024 | .035 | 42.5 | 62.0 |
| | | | (values obtained)* | | (ash calculated)** | |
| C112 | Not known | Normal | .031 | .017 | 50.0 | 27.5 |
| C111 | Not known | Normal | .018 | .008 | 40.5 | 17.0 |
| D29 | Not known | Normal | .096 | .039 | 53.3 | 23.0 |
| D31 | Not known | Normal | .078 | — | 42.6 | — |

* From Table IV.

** Since actual ash weights could not be determined (a limitation of the procedure), they have been calculated by the formula:

$$\frac{\text{ash weight}}{\text{dry weight}} = 0.32$$

A consideration of the anionic composition of animals is necessarily limited; quantitative determinations for phosphate and carbonate were made only on normal animals. Nevertheless, it is possible to suggest certain limitations which are imposed by assuming that calcium is bound primarily only to one or the other, or both, of these anions under all conditions. In Table XI calculations are presented for the phosphate and carbonate contents of a series of normal and destalked animals; these calculations are based on the ionic calcium values which were actually obtained (Tables IV and IX), expressed as oxides of calcium carbonate and calcium phosphate. In each case it was assumed that the calcium is present entirely in the form of these two compounds. In addition, the calculated oxides are expressed as percentages of the ash.

An examination of Table XI indicates that approximately 70% of the ash can be attributed to the oxides of these compounds; about 46% is from carbonate and 23% comes from phosphate. The remaining 30% of the ash probably consists of oxides of silicon, magnesium, aluminum, iron and sulfur since these elements normally occur in crustacean cuticles (Clark and Wheeler, 1922). If the calcium content of *Uca pugilator,* seven days after ecdysis, is assumed to be entirely of calcium carbonate, the oxides which are obtained by incineration will account for 43% of the ash; if the calcium content is assumed to consist completely of calcium phosphate, the resulting oxides would account for 62% of the ash. In contrast to this situation, destalked animals which were analyzed 17 days after ecdysis showed a maximum of 28% ash from carbonate and a maximum of 41% ash from phosphate. From these facts, it would appear quite clear that some other elements were responsible for the remaining ash. If the calcium compound were considered as calcium sulfate, it would account for approximately 70% of the ash. It is known that the sulfur content of lobsters is considerably higher in older animals than in younger animals (Richards, 1951). The possibility exists that the unknown ash component of destalked, postmolt animals contains a high percentage of sulfur; further investigations on the nature of the ash are needed and may be of considerable significance in attempting to explain the role of the sinus gland in mineral metabolism.

That there is an alteration of the calcium : phosphorus ratio in freshly molted animals as compared to premolt animals has been shown by Robertson (1937); his results with *Carcinus maenas* show the integument of normal, freshly molted animals to be considerably higher in phosphorus than in premolt animals. Expressing his values as the ratio of the percentage of calcium to the percentage of phosphorus, it is seen that premolt animals have a ratio of about 9.5 as compared with values of 0.9 shortly after ecdysis, and 1.7 within a week after ecdysis. On the basis of calculations in Table XI, it is not unlikely that a similar situation may occur in *U. pugilator.*

The results of Edwards (1950) with *U. pugnax* are interpreted by that investigator as positive evidence for an increase in weight subsequent to bilateral eyestalk removal. Of a total of ten animals used for one experiment, six showed a weight increase within two days after the operation. However, if the percentage changes in weights of these six animals are expressed as the averages for two, three and seven days after destalking, increases of 3.0%, 3.0% and 5.5%, respectively, are obtained. In contrast, and not discussed by Edwards, similar calculations for animals which showed a decrease in weight after destalking indicate average decreases of 0.5%, 14.4%, 6.3% and 9.3% at the end of one, two, three and eight days, respectively. Yet, on the basis of the data, Edwards concludes that eyestalk removal leads to an increase in weight.

An inspection of Table V might lead one to believe that destalked animals increase in weight. At the end of six, seven, nine and ten days, the percentage increases for five animals are, respectively, 0.7%, 1.6%, 0.3% and 1.9%. However, it should be remembered that the daily fluctuations of wet weights are sufficient to account for at least a 2% variation which may be considered either as an increase or decrease of the wet weight. While the range of fluctuations in weight of a destalked animal is less than that of a normal animal, it is possible to demonstrate similar variations. Until additional studies conducted over a greater period of time are made, it seems necessary to conclude that eyestalk removal has had little effect on the wet weight of *U. pugilator,* at least during a ten-day period following the removal.

That water metabolism is in some manner influenced by a hormone source in the eyestalk has some experimental verification. Data are inadequate for a thorough consideration of this aspect; nevertheless, they suggest a possible role of the sinus gland controlling the rhythmical fluctuation in weight. Normal postmolt animals appear to exhibit a diurnal fluctuation in wet weight (Figs. 7a and 7b). Normal premolt animals also show diurnal fluctuations, but as ecdysis is approached, the fluctuations appear to become arrhythmic. Although destalked premolt animals show minor fluctuations, they were not of a rhythmical character. It will be recalled that these rhythmical fluctuations in wet weight were observed only in animals which were kept in running sea water at the Marine Biological Laboratory. Such a rhythm was not observed in animals which were kept in finger bowls with either sea water or distilled water; under these conditions the sea water was subject to evaporation, while neither medium was aerated to the extent of normally running sea water. These differences may well account for the observed absence of a rhythm under these conditions.

An hypothesis of a rhythm in water-uptake, controlled by the sinus gland-X organ complex and with the phase of wet-weight change as described in this report, would suggest a daily fluctuation in blood calcium concentration of normal animals. If such were the case, it would support an assumption made by Edwards (1950), although the mechanism of control would not be that suggested by Edwards. Finally, it would explain why a more pronounced rhythm is seen in normal postmolt animals than in animals immediately prior to ecdysis, since it is generally thought that the titer of sinus gland hormone is lower just before ecdysis than after ecdysis.

It was observed that normal animals showed no increase in carapace width during a ten-day period following ecdysis, in contrast to the situation in destalked forms (Table VIII). The apparent failure of normal animals to assume greater dimensions during the immediate postmolt period has a number of possible explanations:

1. The environmental factors which prevail in the normal molting habitat may be significantly different from those existing in the laboratory, and may be favorable for a growth response. Normally, animals in the field presumably have an adequate food supply at their disposal; such may not have been the case in the laboratory, since the only food available to the animals was that which was brought in by the running sea water. Changes in the chemical composition of sea water in the salt marshes, effected by drainage, may well exist and be responsible for differences from the conditions in the sea water brought into the laboratory. Following summer rains, a hypotonic condition might have an effect on the amount of water which is absorbed by an animal at ecdysis. The sea water which is pumped into the laboratories at the Marine Biological Laboratory comes from an area which is not subject to any appreciable variation.

2. Since the yearly cycle for *U. pugilator* has not been observed, a situation may exist which is similar to that of certain crayfish. Scudamore (1947) found that

*Cambarus* underwent two yearly molts, growth occurring to a much greater extent in the late summer molt than in the spring molt. Inasmuch as the frequency of molting in normal animals was extremely low during the July-August period, there may well be normally an early summer molt at which time animals exhibit substantial increases in carapace width.

3. Another possibility, and one which is realized in some crustaceans, is that a dimensional increase following ecdysis may be considerably reduced or even lacking under certain conditions. Lloyd and Yonge (1947) found that the growth rate of *Crangon vulgaris* declines as the animals approach maturity and that females which carried eggs showed no increase in length after ecdysis. Hoglund (1943) reported that molt without growth occurs in *Leander squilla* during the winter. Inasmuch as the normal animals on which measurements were taken during the present investigations probably represent adult individuals, it is possible that they had already attained their maximum dimensions. If such an hypothesis is correct, then any additional molts which might occur after such maximal dimensions have been established may serve only to facilitate the regeneration of lost appendages or the repair of damaged portions of the cuticle.

## SUMMARY

1. A transitional series of color changes which occurs during the premolt period of normal animals is described. These changes have been arbitrarily assigned to five stages and the duration of each stage measured. The last four of these are associated with hypodermal activity anticipating ecdysis.

2. The inorganic constituents of normal and destalked animals are compared for the premolt and postmolt condition.

3. Certain physical aspects of ecdysis are treated in a quantitative manner. These include the absorption of water and dimensional changes.

4. The results suggest that the sinus gland-X organ complex plays a role in the regulation of water metabolism and in the metabolism of inorganic constituents.

5. Evidence is presented for a diurnal rhythm of water-uptake under the control of the sinus gland-X organ complex.

6. The possibility that molt and dimensional increase are separate factors under certain conditions is discussed.

## LITERATURE CITED

Abramowitz, A. A., and R. K. Abramowitz, 1940. Moulting, growth, and survival after eyestalk removal in *Uca pugilator*. *Biol. Bull.*, **78**: 179–188.

Baumberger, J. P., and J. M. D. Olmsted, 1928. Changes in the osmotic pressure and water content of crabs during the molt cycle. *Physiol. Zool.*, **1**: 531–534.

Brown, F. A., Jr., 1952. The action of hormones in plants and invertebrates. Ed. by K. V. Thiman. Academic Press Inc., New York.

Brown, F. A., Jr., and O. Cunningham, 1939. Influence of sinus gland of crustaceans on normal viability and ecdysis. *Biol. Bull.*, **77**: 104–114.

Clark, F. W., and W. C. Wheeler, 1922. The inorganic constituents of marine invertebrates. U. S. Geol. Survey, Prof. paper no. 124.

Drach, P., 1939. Mue et cycle d'intermue chez les crustaces decapodes. *Ann. Inst. Oceanogr.*, **19**: 103–391.

Edwards, G. A., 1950. The influence of eyestalk removal on the metabolism of the fiddler crab. *Physiol. Comp. et Oecol.*, **2**: 34–50.

GUYSELMAN, J. B., 1950. An investigation of certain aspects of calcium metabolism in *Uca pugilator*. *Anat. Rec.,* **108**: 116.

GUYSELMAN, J. B., 1951. A new method for determining calcium concentrations in biological materials. *Biol. Bull.,* **101**: 222.

HOGLUND, N., 1943. On the biology and larval development of *Leander squilla* (L.) forma *typica* de Man. *Sven. Hydro.-Biol. Komm. Skr., Ny Ser., Biol., Vol. II, Nr. 6.*

KINCAID, F. D., AND B. T. SCHEER, 1952. Hormonal control of metabolism in crustaceans. IV. Relation of tissue composition of *Hemigrapsus nudus* to intermolt cycle and sinus gland. *Physiol. Zool.,* **25**: 372–380.

KLEINHOLZ, L. H., AND E. BOURQUIN, 1941a. Effects of eyestalk removal on decapod crustaceans. *Proc. Nat. Acad. Sci.,* **27**: 145–149.

KLEINHOLZ, L. H., AND E. BOURQUIN, 1941b. Molting and calcium deposition in decapod crustaceans. *J. Cell. Comp. Physiol.,* **18**: 101–107.

KOLLER, G., 1930. Weitere Untersuchungen über Farbwechsel und Farbwechselhormone bei *Crangon vulgaris. Zeitschr. f. vergl. Physiol.,* **12**: 632–667.

LLOYD, A. J., AND C. M. YONGE, 1947. The biology of *Crangon vulgaris* L. in the Bristol channel and Severn estuary. *J. Mar. Biol. Assoc.,* **26**: 626–661.

MALUF, N. S. R., 1940. The uptake of inorganic electrolytes by the crayfish. *J. Gen. Physiol.,* **25**: 1–28.

NUMANOI, H., 1939. Behavior of blood calcium in the formation of gastroliths in some decapod crustaceans. *Jap. J. Zool.,* **8**: 357–363.

PLANKEMANN, H., 1935. Beitrage zur Physiologie der Garneellenhautung. *Schr. naturw. Vereins Schleswig-Holstein,* **21**: 195–216.

ROBERTSON, J. D., 1937. Some features of the calcium metabolism of the shore crab (*Carcinus maenas* Pennant). *Proc. Roy. Soc. London, Ser. B,* **124**: 162–182.

ROBERTSON, J. D., 1941. The function and metabolism of calcium in the invertebrata. *Biol. Rev.,* **16**: 106–133.

RICHARDS, A. G., 1951. The integument of arthropods. Univ. of Minn. Press, Minneapolis.

SCUDAMORE, H. H., 1947. The influence of the sinus glands upon molting and associated changes in the crayfish. *Physiol. Zool.,* **20**: 187–208.

SMITH, R. I., 1940. Studies on the effects of eyestalk removal upon young crayfish (*Cambarus clarkii* Girard). *Biol. Bull.,* **79**: 145–152.

# RESPIRATION AND IODINE UPTAKE IN ASCOPHYLLUM

SALLY KELLY[1, 2]

*Marine Biological Laboratory, Woods Hole, Mass.*

The brown algae are noted for their high iodine content. They have the property of removing iodine from sea water and accumulating it in their cells in concentrations many times that of sea water. This phenomenon of accumulation against a concentration gradient has been observed for several elements in a variety of tissues. There is considerable evidence that aerobic metabolism is necessary for accumulation (Hoagland, 1940), involving the cytochrome system (Robertson and Turner, 1945; Hoagland and Broyer, 1942), and, perhaps, the Krebs cycle (Machlis, 1944).

Since the mechanism of iodine uptake by the algae might well parallel the uptake of salts previously investigated, it is of interest to know whether iodine uptake is related to respiration. To determine this, three types of experiments were carried out:

1. General nature of respiration by the use of oxidizable substrates and inhibitors.
2. Effect of these substrates and inhibitors on iodine uptake.
3. Iodine uptake in nitrogen.

## MATERIALS AND METHODS

The brown alga, *Ascophyllum nodosum* (Linn.) LeJolis was selected as a suitable species for respiration and iodine uptake measurements because (1) it accumulates iodine; (2) reproducible samples could be obtained from day to day by cutting segments; (3) it remains vegetative in the locality used throughout the summer season; (4) it is readily available.

*Respiratory measurements.* The alga was gathered in the late afternoon, sliced transversely into segments 0.25 mm. thick with a razor blade, and washed overnight in darkness in a running sea water aquarium at a temperature of 18 to 22° C. Segments of this thickness had a respiratory rate of 0.72 $\mu$l. $O_2$ per hour per mg. dry weight (103 $\mu$l. $O_2$ per hour per mg. nitrogen), a rate considerably higher than segments one and ten mm. in thickness (Table I). This suggests that penetration of oxygen into the tissue is an important factor in determining the absolute respiratory rate of this alga. The segments were cut from the distal region between the first and second air bladders; the respiratory rate of segments from this region was lower than that of segments from younger tissue and higher than that of segments from older tissue (Table II). This is evidence that a respiratory gradient exists in this species. Fifty to 400 segments were introduced into each flask, the number being constant in any one experiment. When the latter number was employed,

---

[1] The author's thanks are due the following: the Lalor Foundation for its generous fellowship program; Dr. N. A. Baily for assistance in handling radioactive materials; Dr. E. S. G. Barron, Dr. M. Doty and Dr. D. Mazia for suggestions during the course of experiments.

[2] Address: Div. Labs. and Research, N. Y. State Department of Health, Albany, N. Y.

Table I

*Respiratory rate of segments of various thicknesses. Each figure is the average of 2 to 4 determinations. Tissue starved*

| Thickness in mm. | No. segments in flask | μl. $O_2$ per hour | | |
|---|---|---|---|---|
| | | Per no. segments | Per mg. D.W. | Per mg. N |
| 0.25 | 400 | 84.4 | 0.72 | 103 |
| 1.00 | 100 | 48.3 | 0.57 | 82 |
| 10.0 | 10 | 49.4 | 0.38 | 72 |

the number was determined volumetrically. The respiratory rate was proportional to the number of segments used, indicating that oxygen in the flask was not limiting.

Measurements of oxygen uptake were made in darkness in standard Warburg flasks of approximately 15 ml. volume, maintained in a water bath at 25° C. and shaken at 80 r.p.m. Manometer readings were made every half hour after an equilibration period of 30 minutes. Oxygen uptake was measured as the amount of oxygen taken up by the segments during the entire experimental period of three hours. In the preliminary experiments comparing segments of various thickness and from tissue of different ages, the calculations were based also on dry weight and total nitrogen content of the segments. In general, the basal medium consisted of van't Hoff sea water minus calcium.

The effect of the following substances on respiration was measured: sucrose, mannose, succinic acid, malic acid, sodium pyruvate, malonic acid, sodium azide, iodoacetic acid, sodium fluoride, and potassium cyanide. The experiments with sucrose, mannose, azide, iodoacetic acid and cyanide were carried out at pH 6.8, with segments in van't Hoff sea water solution minus calcium as controls. The experiments with succinic, malic, pyruvic and malonic acids and fluoride were carried out in phosphate-citrate buffers of pH 4.5, 5.5 and 6.5, with segments in buffers of pH range 4.5 to 6.5 as controls. The buffers were made up, and the substances tested dissolved, in van't Hoff sea water solution minus calcium. The respiratory rate was 15 per cent less in van't Hoff sea water than in natural sea water; at pH 6.5 (phosphate-citrate buffer) the rate was decreased an additional 15 per cent.

Table II

*Respiratory rate of segments of different ages. Each figure is the average of 2 to 3 determinations*

| Age of tissue | μl. $O_2$ per hour | | | |
|---|---|---|---|---|
| | Starved | | Not Starved | |
| | Per mg. D.W. | Per mg. N | Per mg. D.W. | Per mg. N |
| Youngest | 1.07 | 200 | 1.52 | 659 |
| Intermediate | 0.60 | 109 | 1.19 | 361 |
| Old | 0.23 | 69 | 0.71 | 380 |

TABLE III

*Effect of sucrose and glucose on respiration. Each figure is the average of 2 to 4 determinations. Tissue starved*

| Concentration in $M$ | Per cent change over control Sucrose | Per cent change over control Glucose |
|---|---|---|
| 0.02 | 8 | 0 |
| 0.05 | 20 | 0 |
| 0.10 | − 2 | 0 |
| 0.20 | − 7 | 15 |

*Iodine uptake measurements.* Segments for iodine uptake measurements differed from those used in respiration studies in being one cm. in thickness. Despite the difference in respiratory rate (Table I), the departure was made to obtain a more uniform counting geometry of iodine taken up than was possible with the thinly sliced segments. They were, however, similarly gathered and washed. The segments were placed in 25 ml. rubber-stoppered Erlenmeyer flasks (10 to a flask) containing 3 ml. of van't Hoff sea water solution to which had been added potassium iodide to give a concentration of 0.05 p.p.m. iodide (the accepted concentration in natural sea water). Iodine$^{131}$, supplied in $NaHSO_3$, was added to the solution in amounts giving approximately 14 $\mu$c. per experimental flask. The flasks were rotated slowly on a turntable.

The iodine$^{131}$ removed by the segments during the experimental period (3 to 6 hours) was determined by obtaining counts of the radioactivity present in the seg-

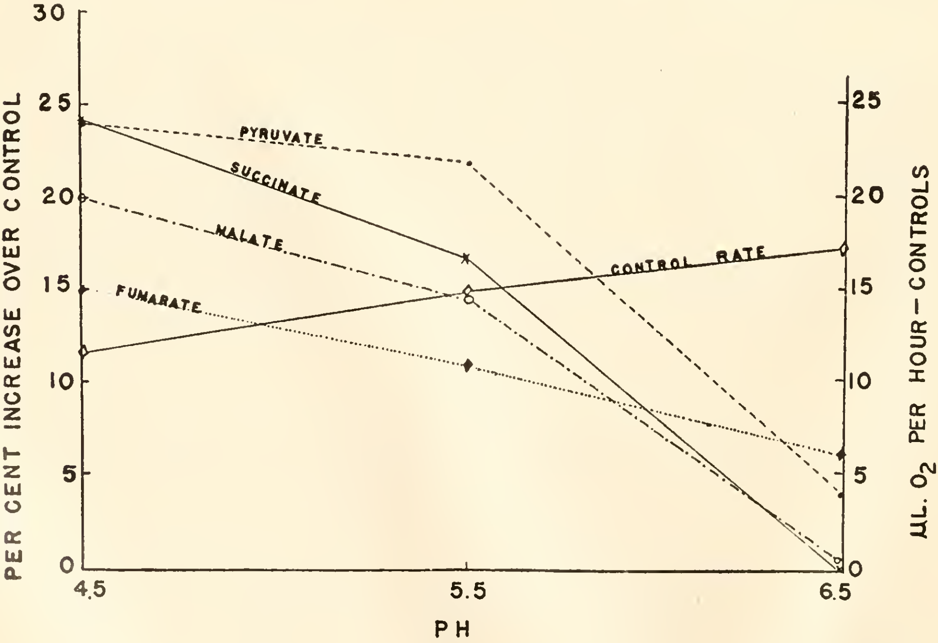


FIGURE 1. Oxygen uptake by Ascophyllum segments in the presence of respiratory intermediates, 0.025 $M$ (except pyruvate, 0.0025 $M$.). Segments in phosphate-citrate buffered van't Hoff sea water minus calcium.

ments and in the solutions before and after the segments were exposed to them. Before the segment counts were made, the segments were removed from the solutions and washed by six successive rinses with a non-radioactive solution, otherwise similar to that in the flasks. Each rinse contained 15 to 20 ml. of solution. By the sixth rinse no further removal of radioactivity from the segments took place. The excess rinse solution was blotted from the segments with a paper towel, and the segments transferred to a counting pan. They were evenly spaced so that a constant geometry was established for each set of segments counted. After counting, they were returned to the radioactive solutions for the next time interval.

Table IV

*Effect of various concentrations of respiratory inhibitors on oxygen uptake and on uptake of iodine*$^{131}$

| Inhibitor and concentration (*M*) | Per cent inhibition | |
|---|---|---|
| | $O_2$ uptake | $I^{131}$ uptake |
| Potassium malonate | | |
| pH 4.5 | | |
| 0.005 | 16 | — |
| 0.010 | 47 | — |
| 0.025 | 87 | 79 |
| 0.050 | 81 | — |
| Sodium azide | | |
| pH 6.5 | | |
| 0.0001 | 8 | 77 |
| 0.001 | 84 | 94 |
| 0.01 | 87 | 96 |
| pH 6.5 | | |
| 0.0001 | — | 71 |
| 0.001 | 13 | 95 |
| 0.01 | 75 | 96 |
| Potassium cyanide | | |
| pH 7.5 | | |
| 0.00001 | 42 | — |
| 0.0001 | 66 | — |
| 0.001 | 71 | — |
| 0.005 | 58 | 42 |
| 0.05 | 61 | 84 |
| Potassium fluoride | | |
| pH 4.5 | | |
| 0.0025 | 29 | — |
| 0.005 | 52 | — |
| 0.025 | 81 | — |

The radioactivity of the solutions was determined by counting two-ml. aliquots of the solutions before and after the segments were exposed to them.

All counting rates were determined by taking at least $2 \times 10^3$ total counts, so that the statistical error is below 2 per cent. The counting rates thus determined were then corrected for background and decay of the iodine$^{131}$.

When a nitrogen atmosphere was desired, the flasks containing the segments were kept in darkness in a vacuum desiccator filled with nitrogen. Before nitrogen was admitted the desiccator was evacuated and the gas passed through alkaline pyrogallol.

The effect of the following substances on iodine uptake was measured: sucrose, glucose, iodoacetic acid, potassium cyanide, and sodium azide. These substances were dissolved in van't Hoff sea water solution, pH 6.8, minus calcium, to which the isotope was added.

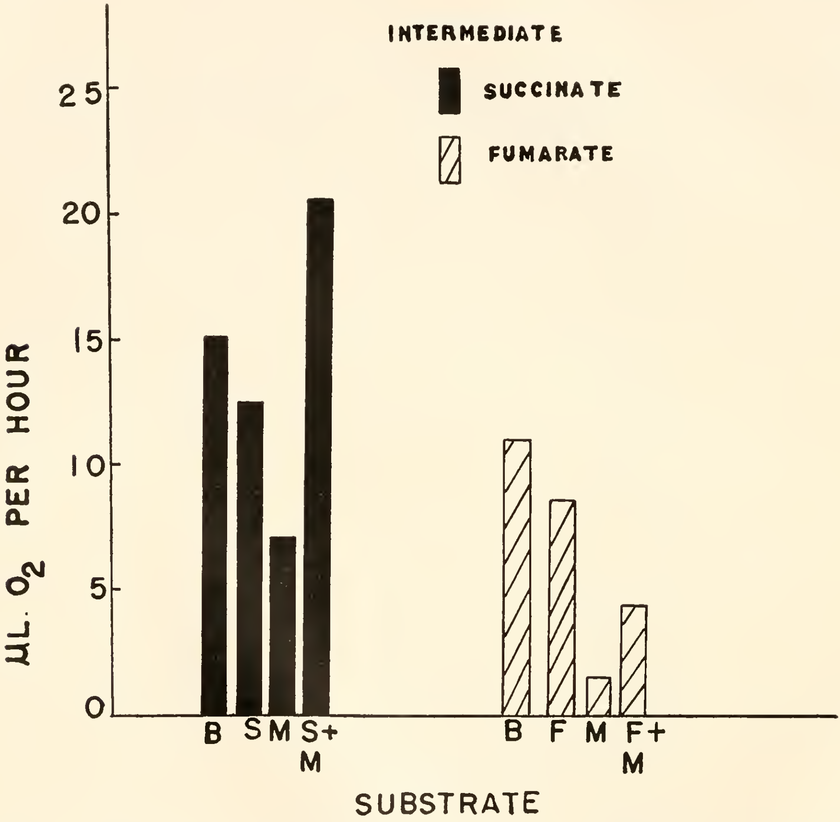


FIGURE 2. Reversal of malonate inhibition of oxygen uptake by Ascophyllum segments. B = basal medium (van't Hoff sea water minus calcium, buffered with phosphate-citrate at pH 4.5); S = succinate; M = malonate; F = fumarate. Concentration of intermediates, 0.025 *M*. Malonate concentration in succinate reversal, 0.01 *M*; in fumarate reversal, 0.05 *M*.

## RESULTS AND DISCUSSION

The respiratory quotient of the Ascophyllum segments was 0.9. Since the enzyme systems involved in Ascophyllum respiration are not known, it was thought feasible to make a survey of the effect of substances that are known to serve as respiratory substrates and inhibitors in other types of cells. Of the sugars tested, sucrose and glucose stimulated oxygen uptake of the segments up to 20 per cent, as indicated in Table III. Mannitol, a storage product of Ascophyllum, had no effect in concentrations of 0.01 and 0.001 *M* and was inhibitory at 0.1 *M*. When the acids, succinic and malic, were tested for their effect on oxygen uptake in van't Hoff sea water at neutral or alkaline pH they stimulated only in high concentrations or after prolonged exposure. When tested at pH 4.5, however, these acids were stimulatory in the concentrations and to the extent given in Figure 1. The respiratory rate of the control at the low pH values was less than at pH 6.5 (Fig. 1). The fact that these acids were effective only at the low pH is in accord with the theory that they enter the cell chiefly in the undissociated state (Baron, 1950; Simon and Beevers, 1951).

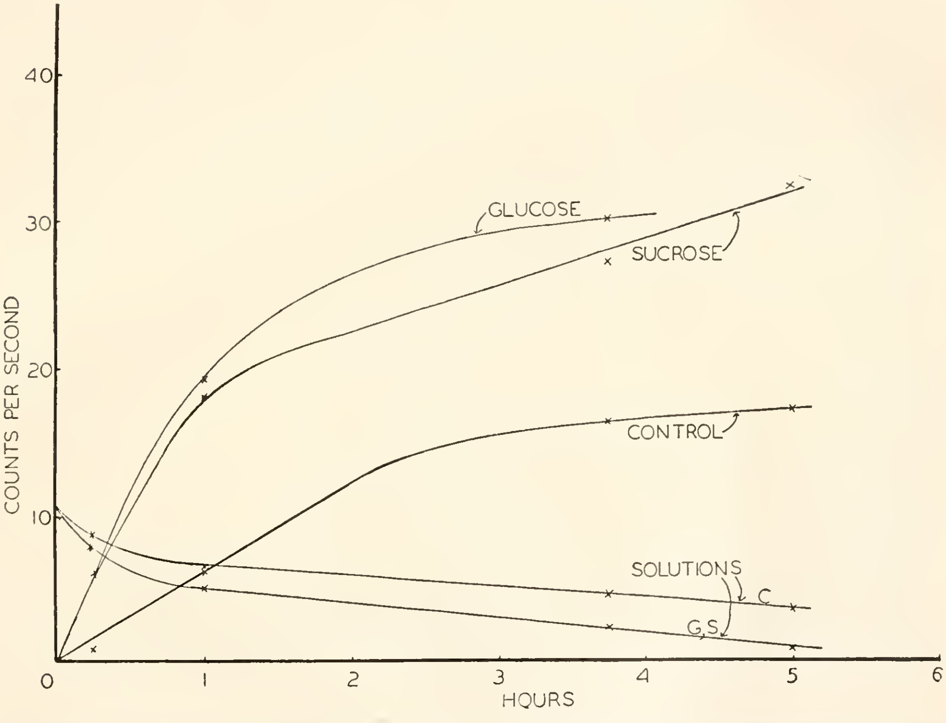


FIGURE 3. Uptake and removal from solution of radioactive iodine by Ascophyllum segments in the presence of added sugars, 0.025 *M*. Segments in van't Hoff sea water minus calcium, containing 0.05 p.p.m. iodide.

The data showing that these acids, known to be intermediates in metabolism of various animal and plant cells, can be oxidized by Ascophyllum segments are suggestive of the presence of a 4-C acid system in this alga.

Additional evidence that this system is present was found in the action of substances inhibitory to respiration of other types of cells: potassium cyanide, sodium azide and iodoacetic acid inhibited oxygen uptake (Table IV). Malonic acid and sodium fluoride inhibited when the pH was lowered to 4.5 but did not inhibit at pH 6.5 (Table IV), again compatible with the theory that these acids enter in the undissociated state. Attempts to reverse the inhibition caused by malonic acid by the addition of succinic and fumaric acids were successful only when the intermediates were present from the start along with the inhibitor (Fig. 2).

These results suggest that heavy metal enzymes, dehydrogenases, and glycolases are functioning in Ascophyllum segments. Anaerobically the segments utilized malic acid, glucose and pyruvic acid, in the order given, while respiring in nitrogen when carbon dioxide production was followed from a bicarbonate buffer.

The amount of iodine$^{131}$ taken up from van't Hoff sea water by the segments increases up to the first hour of exposure to the element; beyond that time no further increase in radioactivity takes place. Since the segments already had iodine in them, having lived in natural sea water until the experimental period, the removal of radioactive iodine has been interpreted as meaning that iodine in the solution is being exchanged for iodine in the segments (Kelly and Baily, 1951). When the ratio of iodine$^{131}$ to iodine$^{128}$ inside the segments becomes equal to the same ratio outside, then net changes in radioactivity cease, indicating an equilibrium has been reached.

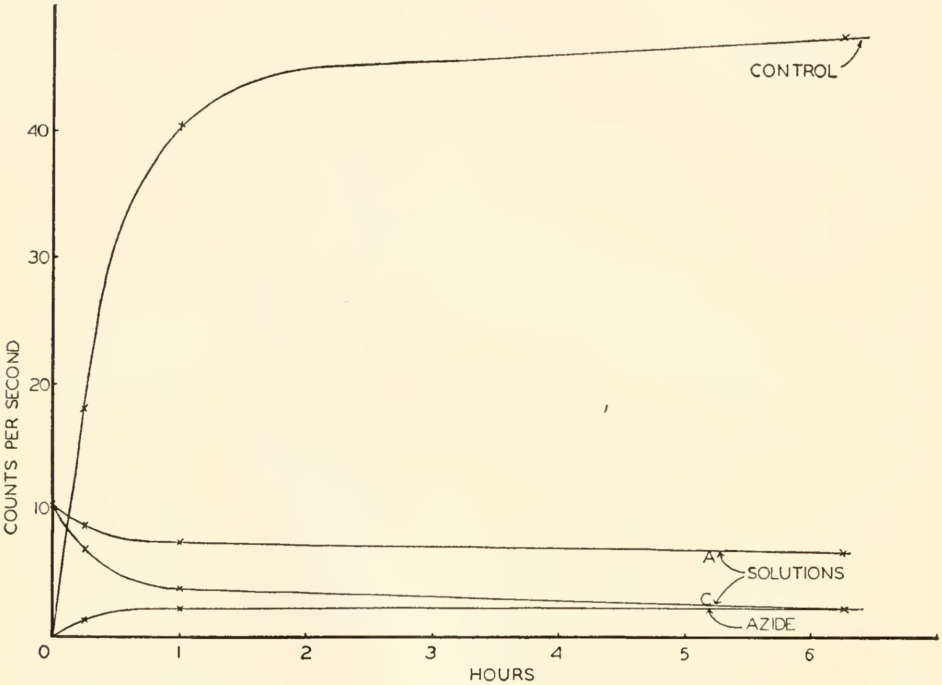


FIGURE 4. Uptake and removal from solution of radioactive iodine by Ascophyllum segments in the presence of sodium azide, 0.001 *M*. Segments in van't Hoff sea water minus calcium, containing 0.05 p.p.m. iodide.

When glucose and sucrose were added to the solution in a concentration of 0.025 *M* the uptake of iodine was stimulated, as measured by the increase in radioactive iodine in the segments and its decrease in the solutions. This stimulation was apparent more or less immediately (Fig. 3). Potassium cyanide, iodoacetic acid and sodium azide inhibited iodine uptake (Fig. 4). A comparison of the inhibitory concentrations and amount of inhibition of both iodine uptake and respiration (Table IV) shows that iodine uptake is more completely inhibited than respiration by comparable concentrations. This suggests that only a fraction of aerobic metabolism is involved in iodine uptake, an interpretation analogous to that given by Commoner and Thimann (1941) to their experiments in which iodoacetic acid completely inhibited growth at concentrations only partially inhibitory to respiration.

The inhibition by cyanide and azide suggests that iodine uptake is an aerobic process. This interpretation is further strengthened by the fact that segments maintained in a nitrogen atmosphere during the period of iodine uptake show a 50 to 75 per cent decrease in amount of iodine uptake.

## SUMMARY

1. Iodine uptake by Ascophyllum was found to be related to the respiratory process on the basis of the following experiments: the uptake of radioactive iodine was stimulated by glucose and sucrose and was inhibited by iodoacetic acid, azide and cyanide. These substances and, in addition, malic and succinic acids and fluoride in turn influenced respiration of the segments, suggesting that they function as respiratory intermediates and inhibitors in the species.

2. Maintaining the segments in nitrogen decreased their radioactive iodine uptake.

## LITERATURE CITED

Baron, L. C. G., 1950. Personal communication.

Commoner, B., and K. V. Thimann, 1941. On the relation between growth and respiration in the Avena coleoptile. *J. Gen. Physiol.*, **24**: 279–296.

Hoagland, D. R., 1940. Salt accumulation by plant cells, with special reference to metabolism and experiments on barley roots. *Cold Spring Harbor Symposium*, **8**: 181–194.

Hoagland, D. R., and T. C. Broyer, 1942. Accumulation of salt and permeability in plant cells. *J. Gen. Physiol.*, **25**: 865–880.

Kelly, S., and N. A. Baily, 1951. The uptake of radioactive iodine by Ascophyllum. *Biol. Bull.*, **100**: 188–190.

Machlis, L., 1944. The influence of some respiratory inhibitors and intermediates on respiration and salt accumulation by excised barley roots. *Amer. J. Bot.*, **31**: 183–192.

Robertson, R. N., and J. S. Turner, 1945. Studies in the metabolism of plant cells. 3. The effects of cyanide on the accumulation of potassium chloride on respiration; the nature of salt respiration. *Aust. J. Exp. Biol. Med. Sci.*, **23**: 63–73.

Simon, E. W., and H. Beevers, 1951. The quantitative relationship between pH and the activity of weak acids and bases in biological experiments. *Science*, **114**: 124–126.

# REPRODUCTIVE CYCLE IN CYPRINA ISLANDICA

VICTOR L. LOOSANOFF

*U. S. Fish and Wildlife Service, Milford, Conn.*

*Cyprina* (Arctica) *islandica* is a clam closely resembling in general appearance the more common species, *Venus mercenaria*. Externally it differs from the latter by having a shaggy, black periostracum which covers the shell. Formerly it was considered to be exclusively a European species but later it was found to be widely distributed on both sides of the North Atlantic. In Europe it ranges from the coasts of England and Norway to the Arctic, while on our Atlantic Coast it is found as far south as Cape Hatteras. However, since it is a cold water species, it is not very abundant south of New England.

A review of the literature shows that many aspects of the biology of *Cyprina islandica* have never been studied. According to Jørgensen (1946) practically nothing is known about the reproduction of this species and, therefore, virtually no literature exists on the subject. Lack of knowledge of the biology of *C. islandica* of our Atlantic Coast is probably due to the difficulty of collecting these clams because they live comparatively far from shore and in deep water. Moreover, since until recently they were not caught commercially, the specimens could not be readily obtained from fishermen.

The situation was changed in 1943 when, in an effort to increase war-time production of sea food, an extensive fishery of *C. islandica* was developed in the waters of Rhode Island and Massachusetts. Since then, samples of these clams have been more easily obtainable. As the fishery expanded and the clams began to appear as a canned product, and in fresh condition, the U. S. Food and Drug Administration in 1944 officially accepted "ocean quahog" as the common name for *C. islandica* (Rhode Island Report, 1945). In this article, for the sake of brevity, we shall call them clams.

Since the beginning of the war-time intensive fishery, new beds of the clams have been discovered. As a rule, these beds are located at a depth ranging from 70 to 120 feet, and are usually confined to either sand and mud or sticky mud (Arcisz and Sandholzer, 1947). Samples of the clams for our studies were collected from such beds located off Point Judith, Rhode Island in approximately 100–120 feet of water. They were collected often enough to record any significant changes in the conditions of the gonads. The clams constituting the samples were adults, several years old, and averaged 3½ to 4 inches in length.

The lack of instruments prevented us from recording the temperature of the water directly over the beds at the time the samples were collected. Fortunately, information on the seasonal temperature changes in that general area is available from the data of Merriman and Warfel (1948), who recorded the bottom temperatures at monthly intervals between September, 1943 and May, 1946 in the area near Point Judith. Because of the proximity of the localities where these temperatures were recorded to our clam beds and because of the strong tidal movements in that general area, it is thought that there should be no pronounced local temperature differences.

Merriman and Warfel (1948) also gave data for bottom salinity of the same general region for the same period. Generally, the salinity was quite steadily maintained between 31.0 and 32.8 p.p.t., suggesting that this is an optimum salinity range for the existence and propagation of *C. islandica.*

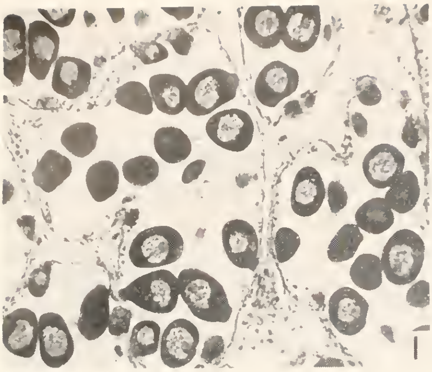
In studying the seasonal gonadal changes of *C. islandica* two approaches were used. The first consisted in histological studies of the material, which led to accurate determination of the condition of the gonads of each individual. Later on this information was used for the second, the statistical, approach, which gave us a quantitative conception of the successive changes in the gonad development during the year and of the progress of spawning during the season. The histological material will be discussed first, while the quantitative data and their analyses will be presented later and summarized in Tables I and II and Figure 13. Figure 13 was made the last figure of the article because of the convenience of not disturbing the continuity of the photomicrographs constituting Figures 1–12 inclusive.

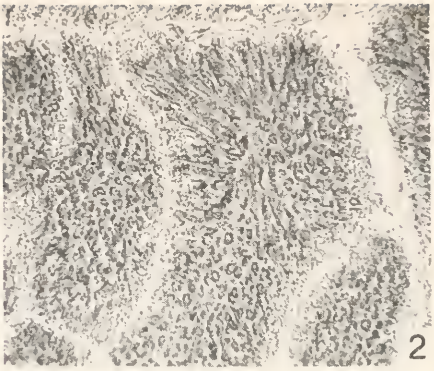
We may begin by considering the condition of the gonads of *C. islandica* just before the beginning of spawning. During the period of our studies this condition was reached by the end of June or early in July, when the temperature was near 13.0° C. Ripe female gonads were characterized at that time by extended follicles containing almost exclusively large, ripe ovocytes (Fig. 1). The walls of the follicles were touching each other, thus leaving virtually no space for the vesicular connective tissue. In males ripe spermatozoa predominated, occupying the largest portion of the follicular spaces, while cells of early stages of spermatogenesis were few in number and were confined to the area near the follicular walls (Fig. 2).

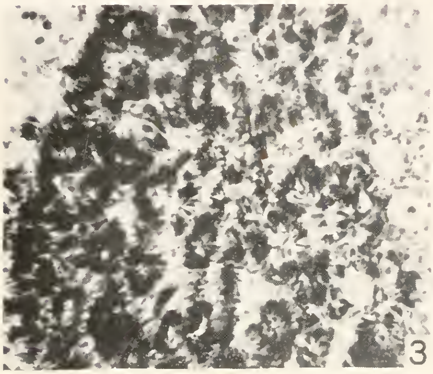
Even a superficial study of the gonads of ripe males often showed masses of "packed" spermatozoa, a condition caused by the arrangement of spermatozoa in the follicles in such a way that their heads were "packed" together creating the impression of a dense band (Fig. 3). A similar phenomenon was observed in *V. mercenaria* (Loosanoff, 1937a, 1937b) and several other lamellibranchs.

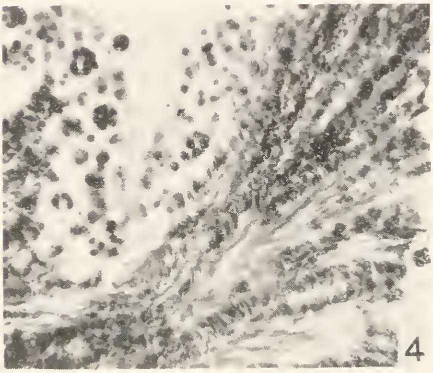
In addition to normal sex cells the follicles of many males also contained numerous multi-celled spherical dark bodies which were usually closer to the periphery of the follicles than to their centers (Fig. 4). Detailed examination of these bodies showed that they were cells of atypical spermatogenesis, noticed by Loosanoff (1937a) in *Venus mercenaria* and described by Coe and Turner (1938) in *Mya arenaria.* These cells apparently develop from the same type of spermatogonia as the normal ones but behave in a strikingly different way, undergoing several nuclear divisions, while still surrounded by the original mass of cytoplasm contained within the same cell membrane. Such abnormal bodies may contain from two to 16 nuclei (Fig. 5). It appears, as was noticed by Coe and Turner (1938), that in some cases such groups of nuclei may break apart and continue further individual development finally reaching the stage of spermatozoa. More often, however, they become pycnotic and are quickly cytolyzed.

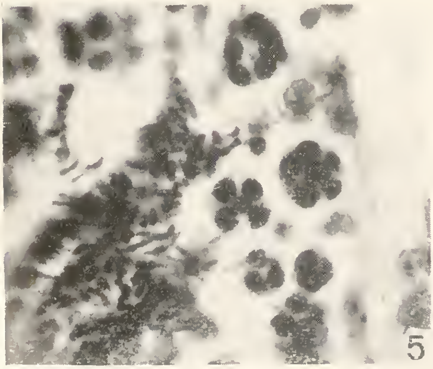
As is usual with lamellibranchs (Nelson, 1928a; Loosanoff, 1937b; Loosanoff, 1942), the entire population of *C. islandica* does not reach ripeness at the same time. We estimate that in the case of these clams only approximately 75 per cent were











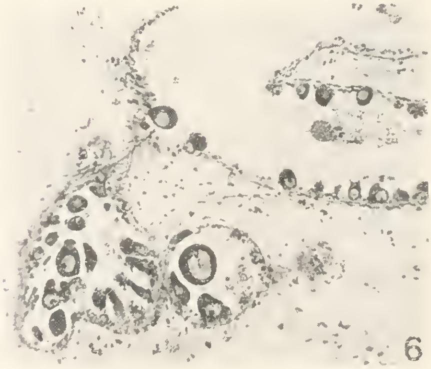


FIGURE 1. Gonad of ripe female. × 112.

FIGURE 2. Gonad of ripe male containing masses of spermatozoa ready to be discharged. × 112.

FIGURE 3. Section of male gonad showing "packed" spermatozoa. × 475.

FIGURE 4. Cells of atypical spermatogenesis occupying the periphery of a follicle containing ripe spermatozoa. × 475.

FIGURE 5. Different groups of cells (spherical) of atypical spermatogenesis. Normal spermatozoa are also present. × 1100.

FIGURE 6. Gonad of female in advanced stages of spawning. × 112.

TABLE I

*Frequencies of occurrence of clams in different stages of sexual development at different dates between March 22 and November 1. Stage A: some ripe cells but generally unripe; Stage B: ripe but had not begun to spawn; Stage C: partly spawned; Stage D: completely spawned*

| Stages | Dates | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3-22 | 4-5 | 4-19 | 5-3 | 5-17 | 5-31 | 6-14 | 6-28 | 7-12 | 7-26 | 8-9 | 8-23 | 9-6 | 9-20 | 10-4 | 10-18 | 11-1 |
| A | 10 | 7 | 8 | | 3 | 4 | | 5 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | 3 | 2 | 2 | | 1 | 3 | | 13 | | 5 | 4 | 8 | 6 | 1 | 0 | 0 | 0 |
| C | 0 | 0 | 0 | | 0 | 0 | | 2 | | 3 | 4 | 10 | 12 | 6 | 3 | 8 | 1 |
| D | 0 | 0 | 0 | | 0 | 0 | | 0 | | 0 | 0 | 1 | 3 | 6 | 4 | 9 | 5 |
| Total | 13 | 9 | 10 | | 4 | 7 | | 20 | | 8 | 8 | 19 | 21 | 13 | 7 | 17 | 6 |

ripe or nearly ripe at the beginning of the spawning season. In the others the gonads still contained a large number of unripe cells. These conditions were especially noticeable in retarded males, the gonads of which contained predominantly the cells of early stages of spermatogenesis, although a few sperm were already present in the centers of some of the follicles.

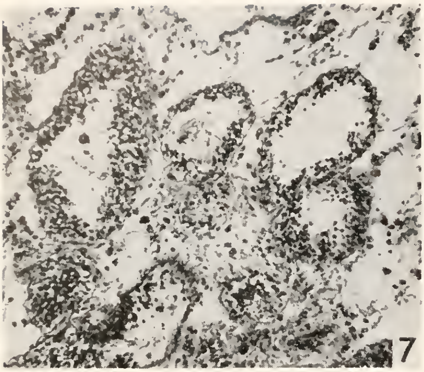
Judging by the condition of the gonads of both sexes, spawning began late in June or early in July when the water temperature over the beds was about 13.5° C. This temperature closely approached that reported by Nelson (1928b) for two other deep-water lamellibranchs, Astarte and Venericardium, which spawn near 12.0° C. Towards the end of July between 30 and 40 per cent of the clams were partly spawned but, as a rule, the quantity of spawn discharged remained small even then, thus indicating that a heavier and more general spawning was yet to follow. This was true because during August spawning continued, involving larger groups of the clam population, and toward the end of this month individuals of both sexes in advanced spawning conditions were becoming more common. In the females constituting this group the gonadal follicles were either empty or contained a few undischarged eggs (Fig. 6). In the males, most of the content of the follicles was discharged but the abnormal cells were still retained in large numbers, being confined to the periphery of the follicles where they were found among the normal cells

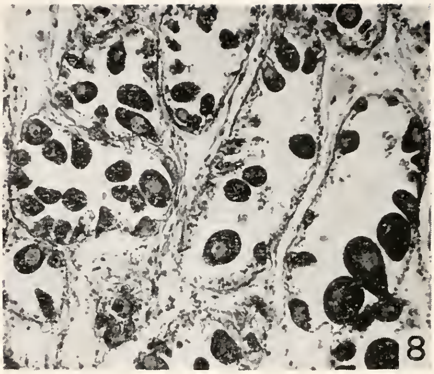
TABLE II

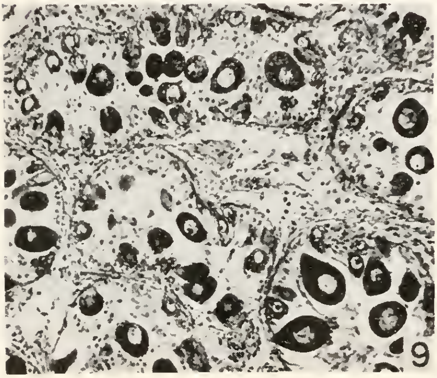
*Cumulative percentage of clams at different stages of sexual development between March 22 and November 1. Stages as defined in Table I*

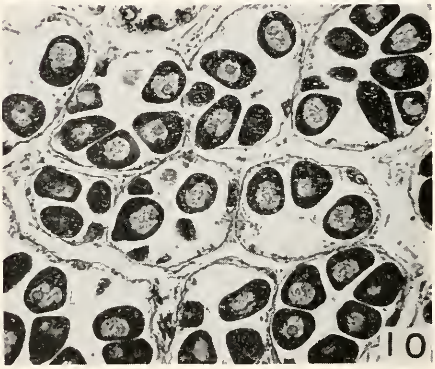
| Stages | Dates | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3-22 | 4-5 | 4-19 | 5-3 | 5-17 | 5-31 | 6-14 | 6-28 | 7-12 | 7-26 | 8-9 | 8-23 | 9-6 | 9-20 | 10-4 | 10-18 | 11-1 |
| B + C + D | 23.1 | 22.2 | 20.0 | | 25.0 | 42.9 | | 75.0 | | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| C + D | 0 | 0 | 0 | | 0 | 0 | | 10.0 | | 37.5 | 50.0 | 57.9 | 71.4 | 92.3 | 100 | 100 | 100 |
| D | 0 | 0 | 0 | | 0 | 0 | | 0 | | 0 | 0 | 5.3 | 14.3 | 46.2 | 57.1 | 52.9 | 83.3 |
| B + C + D (Adjusted*) | 0 | 0 | 0 | | 0 | 20.0 | | 68.8 | | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

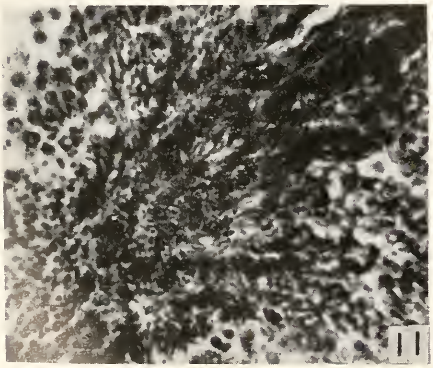
* Per cent in Stages B + C + D after subtraction of 20 per cent of sample size from Stage B and from total.


7


8


9


10


11

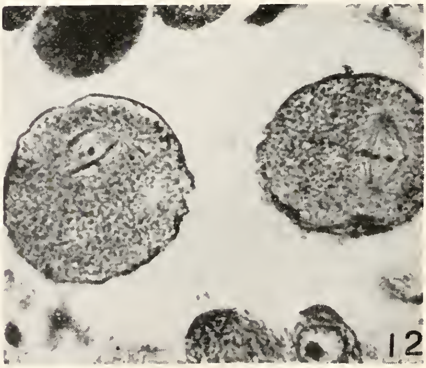

12

FIGURE 7. Almost empty follicles of male in advanced stages of spawning. × 112.

FIGURE 8. Female gonad in late October still containing some old ovocytes but also forming new ones to be discharged next year. × 112.

FIGURE 9. Female gonad in December showing largely small growing ovocytes, but also containing some that are morphologically ripe. × 112.

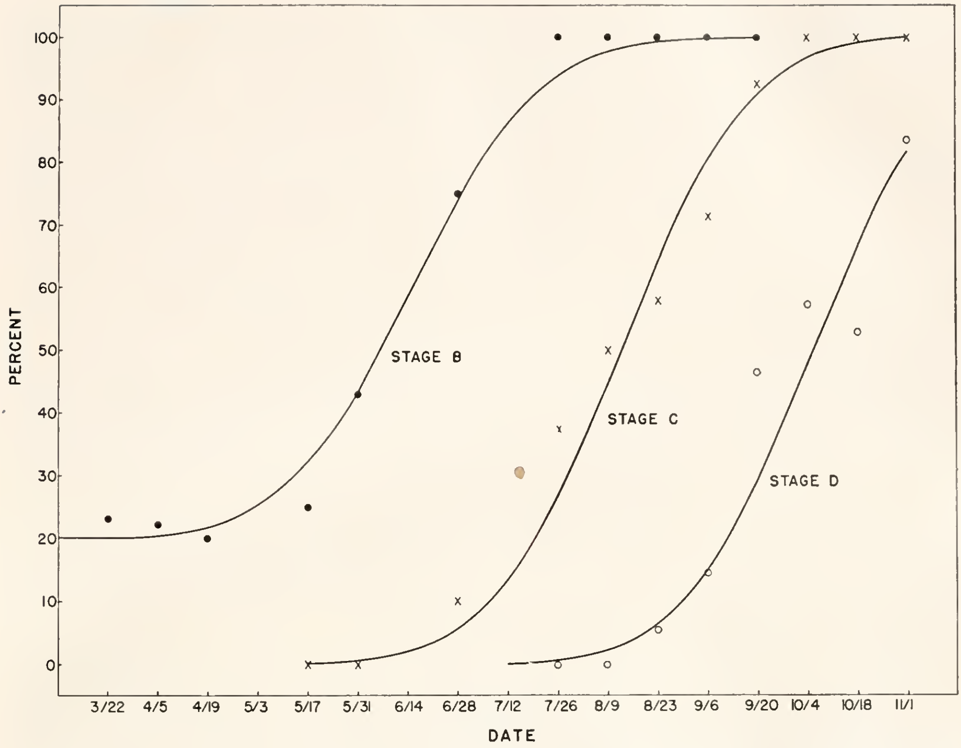


FIGURE 13. Percentage of clams in successive stages of sexual maturity at different times of the year. Stage B: ripe but had not begun to spawn; Stage C: partly spawned; Stage D: completely spawned. Additional explanation in text.

in earlier stages of development (Fig. 7). The emptied follicles were not contracted but remained distended, as was noticed for *V. mercenaria* (Loosanoff, 1937b), and in some instances they gradually became filled with vacuolated cells.

During September, when the water temperature was at its maximum of about 15.0° C., and when partially and fully spawned clams were becoming progressively more numerous, ripe males, showing the so-called "packing" arrangement of the sperm, and ripe but unspawned females still were encountered. In October the temperature of the water was slowly declining but, nevertheless, spawning continued and during the first part of the month was, perhaps, even more active than in September. By the middle of the month the majority of the animals had completed spawning, and towards the end of the month the number of clams with spawn was rapidly diminishing (Tables I and II, Fig. 13).

---

FIGURE 10. Morphologically ripe gonad of a female during late December or in January. × 112.

FIGURE 11. Male gonad in late December or in January already containing ripe spermatozoa. × 475.

FIGURE 12. Eggs ready to be discharged in spawning showing dissolution of the germinal vesicle and the formation of chromosomal spindle. × 475.

During the last part of October and the early part of November the clams passed through the period of recovery consisting in part of resorption of unspawned material. However, as in *V. mercenaria,* there appeared to be no true indifferent period, when the follicles are free of all cells except the indifferent ones as is the case of *C. virginica* (Loosanoff, 1942; Coe, 1943). On the contrary, even then the sex of the clams was well defined, a condition which was especially clearly visible in the females because of the presence in their gonads of large numbers of ovogonia and young ovocytes. Sometimes such ovocytes began to develop before the old ones were discharged (Fig. 8). The presence in the follicles of well defined sex cells during all periods of the annual gonadal cycle indicated, of course, that sex in adult *C. islandica* is quite stable and that sex reversal, as was recently shown for *V. mercenaria* (Loosanoff and Miller, 1950), rarely, if ever, occurs.

After passing through the period of resorption of unspawned material, the clams enter a period of extremely rapid gametogenic activities which continues into December, regardless of the rapidly declining temperature. Some females possessed follicles containing either well grown ovocytes (Fig. 9) or ovocytes that were already so developed that they appeared morphologically ripe (Fig. 10). In males also all stages of development, from relatively unripe males in early stages of spermatogenesis to those the follicles of which contained many spermatozoa, were found (Fig. 11). If some of the material was taken from the spermaries of such advanced males and placed in sea water, the spermatozoa would soon begin swimming in their typical circular motion, indicating the ripeness of the gonads.

During the middle of winter, when the temperature is at its lowest, sometimes approaching only 1.5° C., gametogenesis was slowed down but probably not entirely arrested. Even at such a low temperature the clams remain somewhat active because in a laboratory experiment, in which they were placed in water of about 1.0° C., the clams opened the shells, extended their siphons, and pumped water, thus indicating that they were not hibernating.

With a gradual increase of the temperature in the spring, gametogenesis became progressively more rapid and eventually, by the end of June or early July, a state of physiological and morphological ripeness was achieved by individual clams.

Although the observations on the seasonal gonadal changes of the clams were carried on throughout the entire year, only those data obtained between March 15 and November 1 were analyzed statistically. The late fall and winter conditions were not included in the quantitative analysis. During that period, as already pointed out in the histological discussion, the gonads went through the recovery stage followed by rapid gametogenesis as the result of which approximately 20 per cent of the clams appeared to be morphologically ripe by about March 15.

For the quantitative studies the data were grouped in intervals of two weeks, accepting the mid-point of each of these intervals as the date for all the observations of that group. There were seventeen such intervals but in three of them observations were lacking (Table I).

All clams were assigned to one of four stages depending upon gonad condition. Stage A contained clams which were generally unripe but which, nevertheless, contained some ripe cells. The clams of Stage B were ripe, at least morphologically, but had not begun to spawn. Stage C consisted of partly spawned clams, while Stage D contained those that were completely spawned.

The observed frequencies of the clams in each of the four stages are presented in Table I. Inspection showed that the percentage of clams which have entered Stages B, C or D increases as the season progresses (Table II, Fig. 13). The analysis was based on the provisional assumption that the relation underlying this increasing percentage is the cumulative normal curve, the curves for the different stages of maturity differing only in the dates of their succession. In general, Figure 13 is a comparison between the cumulative percentages obtained from Table II and the theoretical cumulative normal curves which have been postulated.

Since it was found that approximately 20 per cent of the clams possessed gonads that appeared to be morphologically ripe by March 15, the lower limit of the percentage of clams that had entered Stage B at that date was not equal to zero but to about 20 per cent. Our hypothesis was extended to allow for this percentage, only assuming the normal cumulative curve of ripening for the remaining 80 per cent of the population. Accordingly, the number of clams closest to 20 per cent of the total sample in each group was subtracted from Stage B and from the total, giving the values of adjusted percentage in Stages B + C + D (Table II). No adjustment was made in the analysis of Stages C + D and D.

The cumulative percentages given in Table II, adjusted in the case of the class B + C + D, were used to estimate the approximate dates when 50 per cent of the clam population would be expected to have entered Stages B, C or D. Analysis showed that these dates probably fall within ± 3 days of June 15, August 13 and October 6 respectively. Thus, the time interval between the date when 50 per cent of the clams are expected to enter Stage B (ripe but unspawned) and Stage C (partly spawned) was 59 days, and between Stage C and Stage D (completely spawned), 54 days. It should be emphasized once more, however, that the mean date of June 15 applies only to the ripening of 50 per cent of those clams that were not morphologically ripe by March 15. As is evident in Figure 13, the mean date for the population as a whole is about June 4.

The same analysis showed a population standard deviation of 29 days. This means, for example, that 95 per cent of the population would enter a given stage during a period extending from about 60 days before to 60 days after the mean date.

As mentioned above, the standard error of the three critical dates was estimated to be ± 3 days. Larger samples of clams should provide greater precision of these estimates, but at present such refinement does not seem necessary. It was also possible to estimate the goodness of fit of the cumulative normal model, making a separate test of the assumption of equal standard deviations for the three stages of development. After a maximum likelihood fitting by the methods of probit analysis the residual $\chi^2 = 8.68$. We have allowed 28 degrees of freedom in the original $4 \times 14$ table, disregarding those classes with an expected frequency of less than one clam. In fitting the curves given in Figure 13, 18 constants have been used: 14 sample sizes, 3 means and the standard deviation. This leaves 10 degrees of freedom for $\chi^2$, showing a satisfactory agreement. If individual slopes are fitted for the three cumulative curves, the sum of the 3 residual $\chi^2$'s $= 6.77$ with 8 degrees of freedom. The difference, $\chi^2 = 1.91$ with two degrees of freedom, arises from lack of parallelness of the three lines, and is not significant.

Our attempts to induce spawning in ripe *C. islandica* always failed. Methods, such as rapid increase in temperature, addition of suspension of sex products, changes of pH, salinity, etc., produced no results. During the spawning experiments it was found that the clams do not tolerate well temperatures over 27.0° and that at temperatures of 30.0° C. or higher they looked as if anesthetized, a condition manifested by relaxation of the adductor muscles and by expansion of the foot. Usually, subjection to such a high temperature was followed within a few days by a heavy, usually complete, mortality.

Natural unprovoked spawning was observed in only two cases. Once it occurred in the middle of winter, on February 3, 1945, when a single female kept in an aquarium in a cold room, the temperature of which was increased by direct sun to only 9.0° C., discharged a large number of eggs. The actual act of spawning was not seen but the eggs were found in a mass lying next to the clam. The eggs ranged from approximately 80 to 95 $\mu$ in diameter.

The second spawning occurred on July 19, 1948 when clams of both sexes, used several days prior to that date in an unsuccessful spawning experiment, spawned during the night. The temperature at the time of spawning was 22.0° C. The size of the eggs varied between 85 and 90 $\mu$. Some of the eggs were fertilized and showed normal early development.

Attempts to fertilize artificially the eggs stripped from ripe females always failed because such eggs, even after being placed in sea water, still retained intact their germinal vesicles. Under normal conditions the germinal vesicle dissolves while the egg is still in the gonads of the mother clam, just before being discharged in the process of spawning. Immediately upon dissolution of the germinal vesicle a chromosomal spindle is formed (Fig. 12). After that the egg is ready for fertilization. In the stripped eggs, however, such changes do not occur and, therefore, fertilization is impossible. In this way *C. islandica* again closely resembles *V. mercenaria* in which artificial fertilization also cannot be achieved.

In preparing this article I feel especially obliged to my colleague, David W. Calhoun, for his statistical analysis of the data. I also wish to extend my sincere thanks to Charles A. Nomejko for the preparation of the photomicrographs and to William Arcisz for collecting and shipping to me many samples of clam gonads on which this article is based.

## SUMMARY

1. The main period of gametogenesis in both sexes of *Cyprina islandica* occurs in late fall and early winter. In December or January the gonads of some clams present a morphologically ripe appearance, such individuals constituting approximately 20 per cent by March 15. Gametogenesis is slowed down but not entirely arrested during late winter and is resumed at a rapid rate with the spring increase in temperature.

2. Spawning begins near the end of June or early in July when the water temperature is approximately 13.5° C.

3. The dates when 50 per cent of the clam population should be expected to enter the stage of being ripe but not having begun to spawn, the stage of partial spawning, and the stage of completed spawning should fall within ± 3 days of June 4, August 13 and October 6 respectively.

4. In adult *C. islandica* the sexes are separate and well defined even immediately after spawning, thus indicating that sex reversal is uncommon.

5. The eggs of *C. islandica* cannot be artificially fertilized.

## LITERATURE CITED

ARCISZ, W., AND L. A. SANDHOLZER, 1947. A technological study of the ocean quahog fishery. *Com. Fish. Review*, **9**: 1–21.

COE, W. R., 1943. Development of the primary gonads and differentiation of sexuality in *Teredo navalis* and other pelecypod mollusks. *Biol. Bull.*, **84**: 178–186.

COE, W. R., AND HARRY J. TURNER, JR., 1938. Development of the gonads and gametes in the soft-shell clam (*Mya arenaria*). *J. Morph.*, **62**: 91–111.

JØRGENSEN, C. B., 1946. Reproduction and larval development of Danish marine bottom invertebrates. 9. Lamellibranchia. *Medd. Komm. Danmarks Fisk. Havunders., Ser. Plankton,* **4**: 277–311.

LOOSANOFF, V. L., 1937a. Spermatogenesis in the hard-shell clam (*Venus mercenaria* Linnaeus). *Yale J. Biol. Med.*, **9**: 437–442.

LOOSANOFF, V. L., 1937b. Seasonal gonadal changes of adult clams, *Venus mercenaria* (L.). *Biol. Bull.*, **72**: 406–416.

LOOSANOFF, V. L., 1942. Seasonal gonadal changes in adult oysters, *O. virginica,* of Long Island Sound. *Biol. Bull.*, **82**: 195–206.

LOOSANOFF, V. L., AND W. S. MILLER, 1950. On sex reversal in adult clams, *Venus mercenaria. Anat. Rec.*, **108**: 131–132.

MERRIMAN, DANIEL, AND HERBERT E. WARFEL, 1948. Studies on the marine resources of southern New England. *Bull. Bingham Oceanogr. Coll.*, **11**: 131–164.

NELSON, T. C., 1928a. Relation of spawning of the oyster to temperature. *Ecology*, **9**: 145–154.

NELSON, T. C., 1928b. On the distribution of critical temperatures for spawning and for ciliary activity in bivalve molluscs. *Science*, **67**: 220–221.

RHODE ISLAND DEPARTMENT OF AGRICULTURE AND CONSERVATION, DIVISION OF FISH AND GAME, 1945. The ocean quahog fishery of Rhode Island. Pp. 1–31.

# ACTIVITY IN A CRUSTACEAN GANGLION. I. CARDIO-INHIBITION AND ACCELERATION IN PANULIRUS ARGUS[1]

DONALD M. MAYNARD, JR.[2]

*Harvard University, Cambridge, Mass., and the Bermuda Biological Station for Research, St. George's, Bermuda*

The cardiac nerves of arthropods have been studied from time to time (Krijgsman, 1952), but there has been no extended work on the decapod Crustacea. Much has been done on *Limulus* (Carlson, 1905, 1905–06; Heinbecker, 1933, 1936; Garrey, 1942; Prosser, 1943) which seems to have an analogous system with respect to cardiac ganglion and extrinsic nerves, but the ganglion contains a number of cells and by its consequent complexity is of limited usefulness in determining the mechanism of its action as a pacemaker for the heart beat and its relation to the central nervous system.

The decapods have a relatively simple ganglion, or one containing a limited number of cells (Alexandrowicz, 1932) and relatively few extrinsic fibers connecting the ganglion with the central nervous system. The normal heart beat has been shown to have its origin in a burst of nervous activity in the cardiac ganglion (Welsh and Maynard, 1951). A study of some of the details of this activity has been made and will be presented in another paper. Briefly, the frequency of beat is determined by the frequency of the bursts of nervous activity, and the amplitude of contraction is determined by the number of motor impulses per beat. Each cell, as in *Limulus* (Prosser, 1943), may "fire" a number of times per burst.

The heart beat may be augmented or inhibited, both in amplitude and rate, if the proper extrinsic nerves connecting the ganglion with the central nervous system are stimulated. Wiersma and Novitski (1942) give an excellent account of the qualitative effects of the extrinsic nerves in the crayfish heart, and Smith (1947) studied the crab heart and the pharmacology of the extrinsic nerves. However, no quantitative study of the effects of these nerves has been published. The present investigation was undertaken to determine the effects of varying parameters of stimulation of each of the extrinsic nerves. The cardio-inhibitor is of particular interest, for if its action is upon the ganglion, as seems to be the case, this is a preparation in which an inhibition, possibly analogous to central inhibition, may be studied. The pertinent anatomy was worked out, and a general description, supplementing that of Alexandrowicz (1932), is given.

---

[1] Contribution number 189 from the Bermuda Biological Station for Research, Inc. This work was supported by grants from Harvard University and the Bermuda Biological Station.

[2] Present address: Department of Zoology, University of California, Los Angeles, California.

## MATERIALS AND METHODS

Thirty-eight adult *Panulirus argus* (Latreille), the Bermuda spiny lobster, were used. Antennae and legs of the lobsters were removed, the ventral nerve cord severed at the base of the abdomen to prevent movement, and the animal allowed to bleed. The carapace anterior to the cervical groove was then removed, and the viscera dissected out. Care was taken to prevent leakage of digestive fluids into the cavity, which was washed with sea water upon removal of the organs. The heart was perfused with sea water by means of a cannula inserted into the opening of the ophthalmic artery. After a period of initial irregularity, the heart usually beat regularly and continuously for several hours at room temperature, about 27° C.

For morphological study, the heart or portions of the central nervous system were stained with dilute methylene blue in sea water. The preparation was usually studied fresh, and then fixed in ammonium molybdate (Alexandrowicz, 1932).

In such a preparation, the cardio-inhibitors are exposed on the surface of the abdominal flexors; the first and second cardio-accelerators may be exposed by cutting and lifting the first and second abdominal flexors. The accelerators are also visible for a short distance laterally above the muscles before entering the pericardium.

Silver wire hook electrodes 2.5 mm. apart were used for stimulation. Square stimulus pulses were supplied by a Grass 3-BC stimulator.

The heart contraction was recorded by kymograph. The recording lever was attached to the cannula perfusing the heart rather than to the heart directly.

The percentage of acceleration or inhibition was obtained by dividing the change in beat by the normal rate determined immediately before stimulation. The heart rate was not constant, usually varying plus or minus 10% during a run of several minutes and some 20% over several hours. The average maximum and minimum rates recorded in the normal beat of 11 animals were 71.2 and 47.8 beats per minute. The absolute maximal and minimal rates for these animals were 115 and 29.5 beats per minute. In general, a difference of 10% is significant in determining the effects of the extrinsic nerves.

## RESULTS

### *Anatomy*

The cardiac ganglion (Alexandrowicz, 1932; Welsh, 1939) is similar to those of other marine decapod Crustacea. It is Y-shaped and lies on the inside of the dorsal wall of the heart, crossed by one or two muscle strands. There are five large ganglion cells; four of these are grouped anteriorly, close together at the fork of the "Y", the fifth occurs about half-way down the stem at the junction of prominent side branches. In the posterior half of the stem, there are four smaller cell bodies arranged linearly with more or less equal spacing. The large cell bodies averaged 54 × 84 $\mu$ in fixed preparations, and the small cells, 22 × 43 $\mu$. Fibers from the ganglion cells first run in the central trunk of the ganglion and then branch into the myocardium, ramifying throughout the muscle. They also give off collaterals which form neuropiles within the trunk or extend into the muscle to form arborizations (see Alexandrowicz, 1932).

The cardiac ganglion is connected to the central nervous system by fibers which arise in the thoracic ganglion and reach the heart via the inhibitor or accelerator nerves, the pericardial plexus, and the dorsal nerve (Fig. 1A).

The pair of cardio-inhibitor nerves (CI) arises in the anterior region of the thoracic ganglion. On each side, one of the pair runs forward along the sternal canal to the cephalic apodeme, where it leaves the canal, passes through a small opening or notch in the endophragmal plate, and then runs dorsally along the surface of the abdominal flexors to the anterior border of the pericardium. There it enters the pericardium and joins the lateral pericardial plexus (pp).

The two pairs of cardio-accelerators (CA-1 and CA-2) arise immediately posterior to the cardio-inhibitor in the ganglion. The second is the most anterior of a pair of nerves arising from the base of the nerve to the first pereiopod; the first accelerator is anterior to this, presumably from the region at the base of the third maxilliped nerve. These would correspond to segmental nerves "m" and "l" which were described by Heath (1941) in the crab, *Pugettia*. They run forward along the sternal canal for a short way, but leave it through sinuses in the endophragmal plate in the first and second segmental region, respectively, behind the cardio-inhibitor. Then they run dorsally beneath the abdominal flexors to the pericardium where they enter the lateral pericardial plexus. The posterior cardio-accelerator branches before entering the pericardium, its anterior branch going to the plexus while the posterior branch proceeds to the pericardium sending off twigs to the muscles of the dorsal region and to the ligaments of the heart.

A pair of dorsal nerves (DN) carries the accelerator and inhibitor fibers from the lateral pericardial plexus to the heart. On leaving the plexus, one of the pair may contain as many as 11 methylene blue-stainable fibers; one of these branches off soon after leaving the plexus and enters the dorsal musculature. The others remain until the nerve reaches the lateral suspensory ligaments where the nerve passes through another plexus which ramifies over the ligaments. Here most of the fibers in the dorsal nerve branch and leave until two to four are left which actually enter the heart. These fibers can be traced back through the ligamental plexus (lp) and shown to proceed directly along the nerve from the lateral plexus. They may be considered as cardio-regulators, while the additional number originally in the dorsal nerve appear to be concerned with some function in the ligaments. As shown in Figure 1A, the ligamental plexus is composed of ramifications of branches from the lateral plexus. The posterior ligamental plexus may also receive branches from the posterior branch of the second cardio-accelerator.

The dorsal nerve enters the heart dorso-laterally between the dorsal ostia (Fig. 1A). After a slight swelling at the surface of the myocardium, it usually runs directly to the ganglionic trunk at the level of the third or fourth large cell. There is much variation; the path of the nerve on each side of the heart is usually different, and in some cases small branches are given off to the muscle or there is an anastomosis with nerve branches from the central trunk. It could not be ascertained whether the small branches contain collaterals from the regulator fibers. In the anastomoses, there was no union of the actual nerve fibers; the sheath of connective tissue is merely united.

Alexandrowicz (1932) distinguished thick and thin fibers within the dorsal nerve which he termed System I and System II. In the dorsal nerve of *Panulirus,* a maximum of four fibers, similar in size (5 to 9$\mu$), stained well with methylene

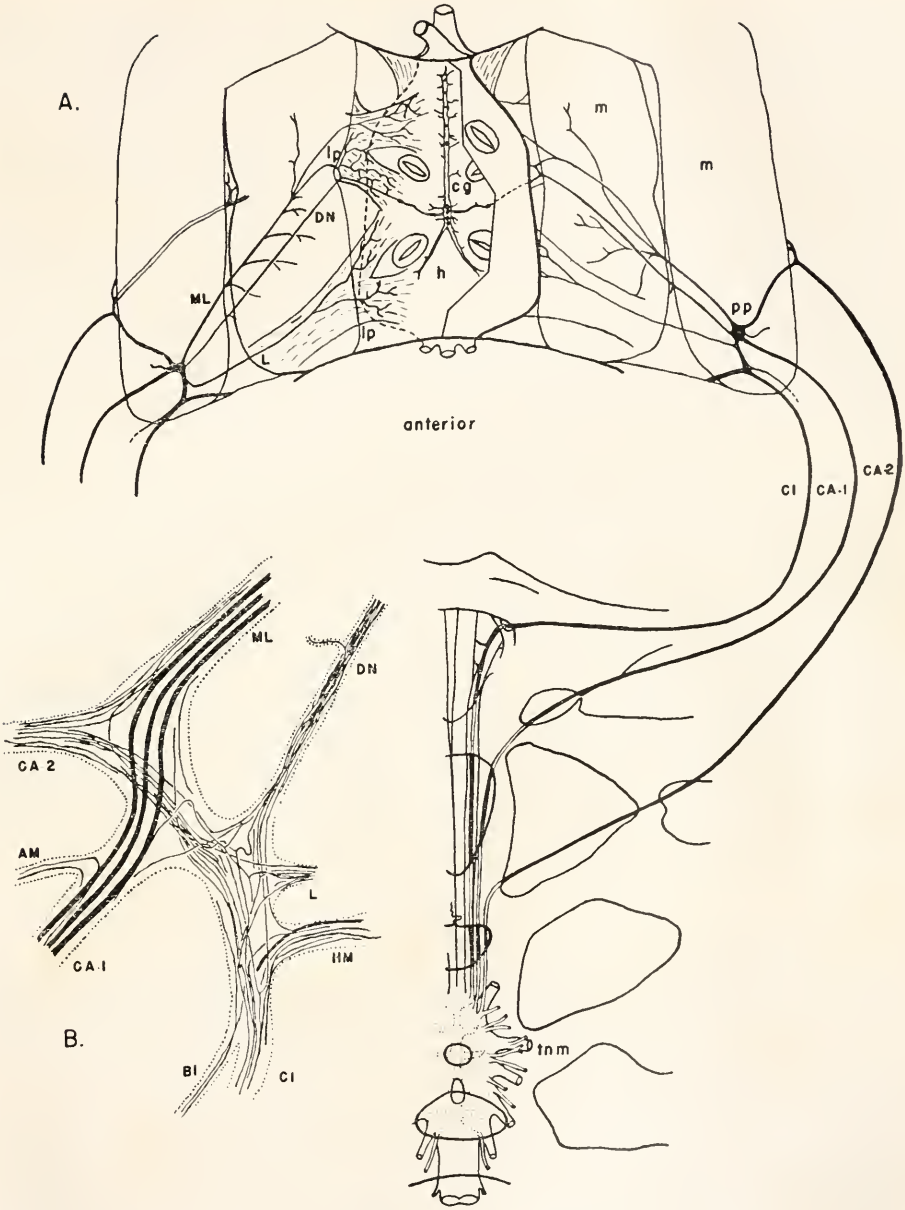


FIGURE 1. Cardiac nerves of *Panulirus argus*. A. Thorax split horizontally and upper-half laid back, giving dorsal view of thoracic ganglion and ventral view of heart. The heart is slightly enlarged with ½ ventral side removed to show cardiac ganglion. B. Left lateral pericardial plexus, enlarged to show nerve fibers. AM, branch to dorsal abdominal muscles; BI, branch from cardio-inhibitor; CA-1, first cardio-accelerator; CA-2, second cardio-accelerator; cg, cardiac ganglion; CI, cardio-inhibitor; DN, dorsal nerve; h, heart; HM, nerve to hypodermis and dorsal muscles; L, ligamental nerve; lp, ligamental plexus; m, muscles; ML, nerve to ligaments and dorsal muscles; pp, lateral pericardial plexus; tnm, thoracic ganglion.

blue. These correspond to System I. Upon entering the ganglion they divide, sending equal processes anteriorly and posteriorly in the trunk. These immediately send out small branches which either form networks over the surface of the large cells or help form neuropiles within the ganglion. The main processes can be traced along the trunk and seen to send out occasional branches. According to Alexandrowicz, such branches end in the muscle close to the endings of the ganglion cell fibers. The regulator fibers would therefore act both in the ganglion on cell bodies and in the muscle.

System II stained only in rare instances when smaller fibers were noted entering the ganglion with System I. They may have also been present but unidentifiable in the general fiber complex of the trunk.

Figure 1B gives semi-diagrammatically the general relationships of the nerve fibers entering and leaving the lateral plexus. There are about nine nerves involved: 1) the cardio-inhibitor, 2) a branch at the base of the cardio-inhibitor which proceeds along it for a distance (BI), 3) the first cardio-accelerator, 4) a branch containing two fibers from the first cardio-accelerator which runs to the abdominal extensor (AM), 5) the second cardio-accelerator, 6) a branch which contains large motor fibers from the first cardio-accelerator and smaller fibers supplying the suspensory ligaments of the heart (ML), 7) the dorsal nerve, 8) the anterior ligamental nerve, running to the anterior suspensory ligaments of the heart, 9) a branch consisting largely of fibers from the cardio-inhibitor which do not cross the plexus but appear to run dorsally into the muscles and hypodermis (HM). The nerve paths may vary greatly, especially (6), which as 'ml' in Figure 1 follows a different course on each side. The number of fibers shown in Figure 1B is not complete, for all did not stain with methylene blue. A number of fibers entering the dorsal nerve branch before entering, while there are some from each of the cardio-accelerators and the cardio-inhibitor which do not branch. It is among these latter that the actual regulator fibers are probably found, while the branching fibers are presumably those which innervate the ligaments of the heart.

## *Physiology*

### 1. *Cardio-inhibitor*

Both the number of active fibers and the frequency or pattern of impulses per fiber presumably act in producing a graded effect on the heart beat. In analysis, therefore, the response of the nerve to stimulation was separated from the response of the heart to the inhibitor nerve.

*a. Excitability of inhibitory fibers.* A frequency was chosen (70 per second) which had been shown to give complete or nearly complete inhibition. This was kept constant and the threshold at varying strengths and pulse durations determined (Fig. 2). The pseudochronaxie was 0.2–0.5 milliseconds, and k value (Hill's time constant), 0.29–0.72. In one case, not in Figure 2, a pseudochronaxie of 1.45 milliseconds was obtained.

The absolute values of the pseudochronaxie, though subject to errors due to polarization of the electrodes, are of the same order of magnitude as others obtained for crustacean nerves (Jasper and Monnier, 1933). The lack of irregularities in the curve indicates that a single physiological unit is being stimulated. At threshhold voltage, the response was often merely a temporary hesitancy in the heart beat, indicating a transitory stimulation at the electrodes.

If either voltage or pulse duration was well above minimum threshold value, increasing the other parameter caused a stepwise, not graded, increase in inhibition. Thus, an incompletely inhibited heart showed no effect as the pulse duration was increased several hundred per cent. Then suddenly a second threshold level was reached and the heart became completely inhibited. Usually there were two such levels, though in one case there were three, and in several instances there was no increase over the first minimum threshold effect. If either parameter was near minimum threshold value, the second level could not be obtained (Fig. 4). It thus has the qualities of a true second threshold.

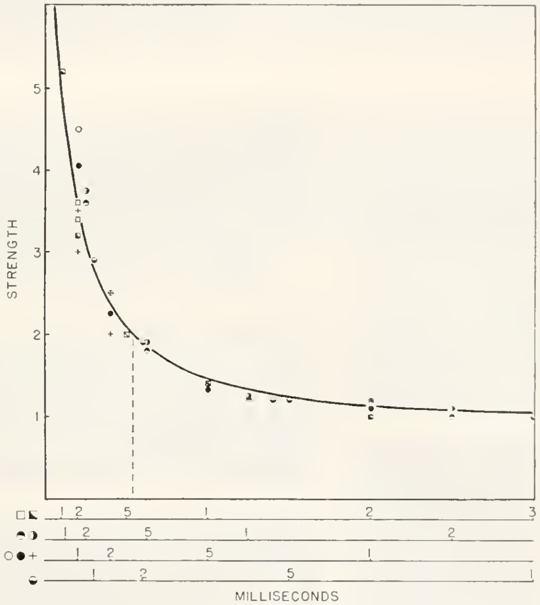


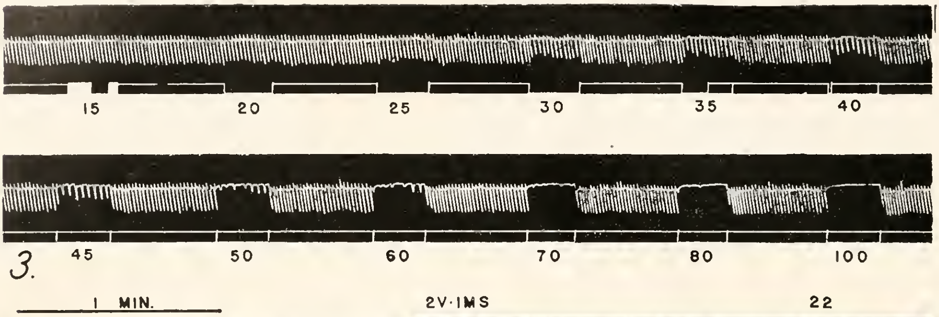
FIGURE 2. Strength-duration curve. Ordinate in multiples of rheobase. ○, cardio-inhibitor; □, first cardio-accelerator; +, second cardio-accelerator.

With increasing voltage and a constant pulse duration, there was a small but definite range (0.05–0.2 volts) at minimum threshold and at each step where there was a gradation before the final level of inhibition was reached. It would appear that in this relatively narrow range the fiber responds to some but not all of the stimuli, but that after the fiber responds to each stimulus, a change in voltage no longer causes increased response in that fiber.

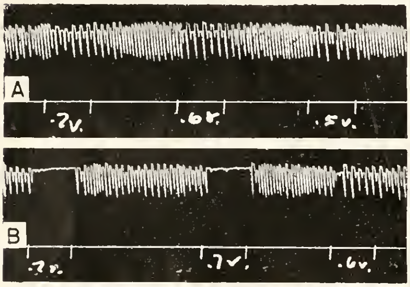
In several instances it was apparent that the fibers running to either the ligamental plexuses or the dorsal musculature were stimulated, for a change in the base line not otherwise associated with the heart beat was obtained upon stimulation.

With possible exceptions at low frequencies of stimulation, repetitive responses to each stimulus did not occur. The regular relation between frequency and effect, the fact that changing pulse duration and voltage did not result in a consequent gradation in effect, and the low pseudochronaxies argue for this conclusion.

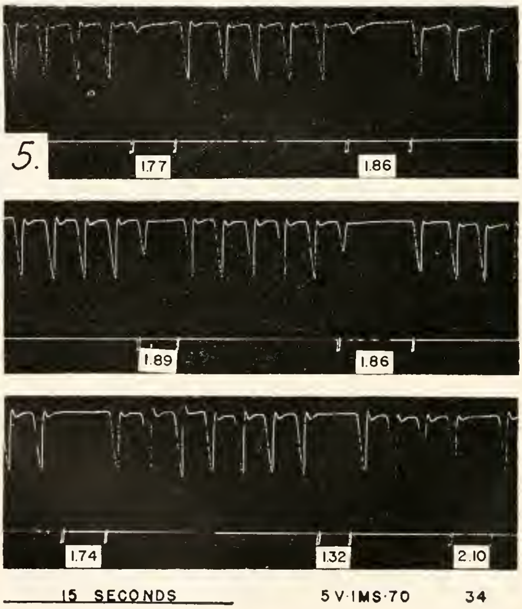
Exhaustion of the cardio-inhibitor was noted in cases of long-continued stimulation. There was either a gradual and irregular, or sudden increase in the heart



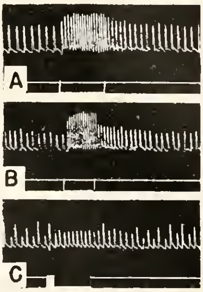
3.



4.



5.



6.

FIGURE 3. Cardio-inhibitor. Frequency varied; stimulus strength, 2 volts, 1 millisecond pulse duration.

FIGURE 4. Cardio-inhibitor; interrelation of pulse duration and stimulus strength. Stimulus frequency, 40 per second, voltage varied. A. Pulse duration, 0.2 milliseconds; B. pulse duration, 0.5 milliseconds. Note alternating beat.

FIGURE 5. Cardio-inhibitor. There is progressively less effect on the beat immediately following the initiation of stimulation as the time between these events is decreased. Stimulation at 70 per second, 5 volts, 1 millisecond.

FIGURE 6. Cardio-accelerator. A. Cardio-accelerator 1; stimulation at 50 per second, 1.4 volts. B. Cardio-accelerator 2; stimulation at 50 per second, 0.5 volts. Note the after-effect. C. Cardio-accelerator 2; stimulation at 15 per second, 5 volts. The alternating beat is abolished upon stimulation, but returns upon the release of stimulus.

beat. Since it was found that the recovery process following accomodation or exhaustion of the cardio-inhibitor was slower or followed a different time course from the accomodation itself (Levin, 1927), a 15-second stimulus period was usually followed by a 30-second rest in determining the stimulus-response curve. This in most cases proved adequate to maintain the nerve in an unexhausted state.

*b. Response of the heart to the inhibitor.* At a given level the percentage of inhibition obtained was characteristically a non-linear function of the frequency of stimulation. In most cases complete inhibition was obtained by stimuli at 100 per second. Exhaustion of the nerve was frequent at frequencies between 80 and 500, and the threshold of stimulation was often raised (Pantin, 1936). The upper limit of the frequency of stimulation appears, when obtained, to be due to the failure of the cardio-inhibitor rather than to some effect at the cardiac ganglion. The minimum frequency producing detectable inhibition was about 10–15 per second (Fig. 3). In several instances stimulation at 15 per second or less had slightly more effect than stimulation at 20 per second.

As the frequency of stimulation was increased, the percentage of inhibition, as measured by decreased frequency or amplitude of beat, increased. Because each of the two or three threshold levels gave a different stimulus-response curve, it was impossible to average the results of all experiments. Values obtained for six animals were grouped roughly according to the pulse duration of the applied stimulus (Fig. 7). Curve 1 is above the first threshold, the pulse duration is rather short. In Curve 2, the second threshold level, the pulses were of long duration, and in Curve 3 are the long pulses of one preparation which also showed threshold levels 1 and 2. With aging the thresholds tended to change, and in several instances the older preparations showed only the first threshold effect.

Usually the inhibited amplitude and frequency remained regular and in some instances served to regularize a normally irregular beat. Occasionally, however, stimulation of the inhibitor caused an irregular, inhibited beat. Since this irregularity was also noted near the nerve threshold, it seems most likely that it was because of the irregularity of the impulses arriving at the ganglion. In certain other instances a beat alternating between a strong and weak contraction (Fig. 4) was initiated. This seems to be a peculiarity of the ganglionic action and was very often noted in old or slightly injured preparations. It is also found in non-nervous but rhythmically active centers such as the molluscan heart (Welsh and Taub, 1950, Fig. 1). Near the frequencies causing complete inhibition it was not unusual to find the heart contractions either quite arrhythmical or of varying strengths. It is not clear whether these, and the occasional escape beats which occurred during otherwise complete inhibition, are due to changes in the threshold of the cardio-inhibitor nerves and consequently the number of impulses arriving at the ganglion, or whether they are due to some change in the threshold of the ganglion's response to the arriving impulses.

Facilitation is present, but the period of facilitation must be quite short. At frequencies of 20 per second, there is some evidence that there is a progressive inhibition during the first few beats. At higher frequencies the heart beat is much too slow to indicate facilitation in the ganglion, and inhibition is immediate. As shown below, a stimulus of 70 per second may produce its maximum effect in less than ⅓ of a second.

If, in a heart beating approximately every two seconds (Fig. 5), a stimulus of 70 per second was applied up to 1.7 seconds after a beat, the next beat was completely inhibited. If, however, the stimulus was applied between 1.7 and 2.1 seconds after a beat, the following heart contraction was only partially inhibited. Thus for a period of ⅓ second, a progressive inhibition in the beat following the initiation of the stimulus was obtained. It would seem most probable that this is accomplished by blocking a portion of the nervous activity in the ganglion, and that the ⅓ second measures the maximum duration of facilitation at this frequency of stimulation.

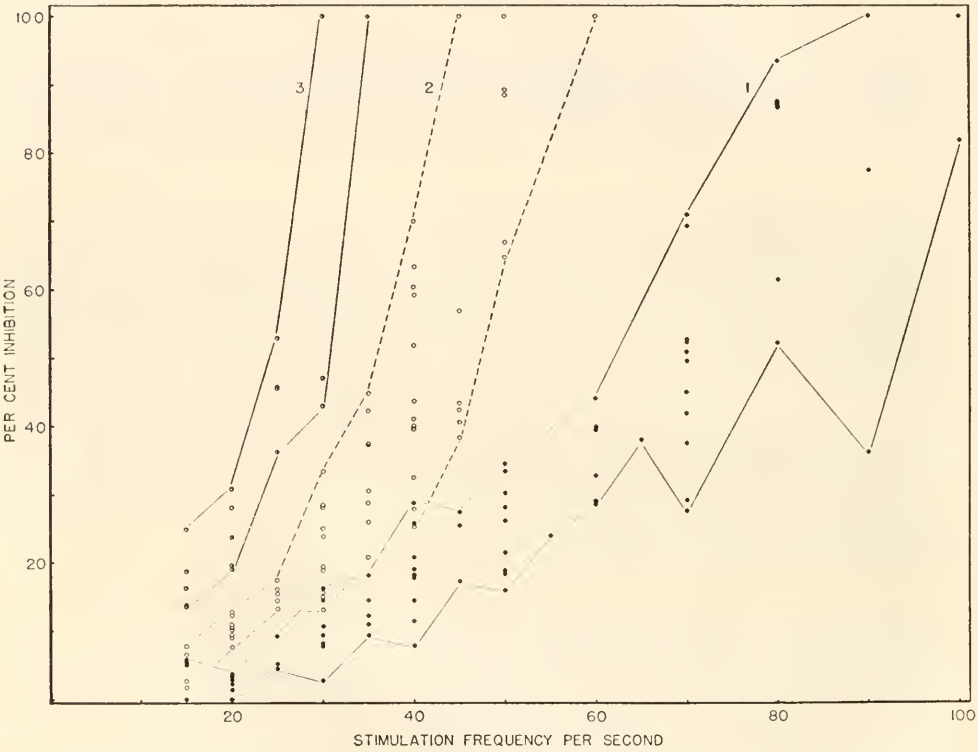


FIGURE 7. Cardio-inhibitor; stimulus-response curves for 6 animals. Curve 1, pulse duration, 0.2 to 5 milliseconds; Curve 2, 1 to 10 milliseconds; Curve 3, 5 to 10 milliseconds. In any one animal there was a sharp division between curves, but the thresholds varied among animals.

Four phenomena may be noted upon cessation of inhibitory stimulation: 1) there may be a series of beats of gradually increasing frequency and amplitude until the normal state is reached; 2) there may be an exceptionally large contraction immediately following the release of inhibition (this may be followed by a period of contractions alternating in amplitude and frequency, or a gradual increase as in (1)); 3) there may be a period of accelerated beat and larger amplitude immediately following inhibition; 4) there may occasionally be no noticeable after-effect. The duration of inhibition does not greatly influence the after-effect.

## 2. *Cardio-accelerators*

The investigation of the cardio-accelerators was not as extensive as that of the cardio-inhibitor. The pseudochronaxies of both cardio-accelerator nerves (Fig. 2) were not significantly different from those obtained for the cardio-inhibitor. A second threshold was found, though not as commonly as with the inhibitor. Comparisons of the thresholds at different frequencies were not made.

From the responses in Figure 6 and the similarity of the shape of the frequency-acceleration curves, it seems fairly clear that the two cardio-accelerators act in the same manner on the heart, though it is not possible to say that they have the same quantitative effect. In Figure 9A twice as many fibers were probably stimulated in the first cardio-accelerator as in the second. A later series of stimuli applied to the first cardio-accelerator produced a curve of acceleration with a maximum reduced to approximately that of the second accelerator.

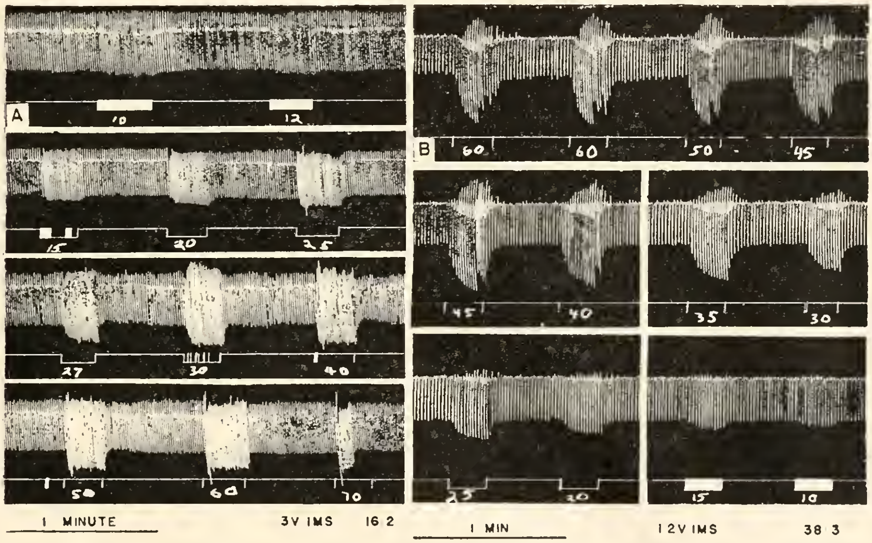


FIGURE 8. Cardio-accelerator; frequency varied. A. Cardio-accelerator 1; stimulation at 3 volts, 1 millisecond. B. Cardio-accelerator 2; stimulation at 1.2 volts, 1 millisecond.

In several instances stimulation of the cardio-accelerators restored a regular beat to an irregular or inactive heart. In such cases, the maximum rate obtained (95–125 per minute) was comparable to the maximum rate obtained from a normally beating heart upon stimulation (74–140 per minute). As Wiersma and Novitski (1942) found, however, the maximum was in many cases correlated with the normal heart rate. The cardio-accelerators usually destroyed an alternating rhythm by equalizing succeeding beats, redistributing a given number of impulses into equal bursts rather than by only increasing the number of impulses in the weaker bursts (Fig. 6).

The effects of varying frequencies of stimulation are shown in Figures 8 and 9.

The amplitude and rate of beat increased in a similar fashion, and there is a maximum effect which is usually reached by stimulation frequencies of 60 per second. No further effect may be obtained with frequencies up to 500 per second. The lower limit of effective frequency varied in the vicinity of 2–5 per second. In a manner similar to stimulation of the cardio-inhibitor, stimuli at 10 per second often were more effective than stimuli at 15 per second. It is not clear whether these responses at the lower frequencies involved repetitive discharges in the nerve.

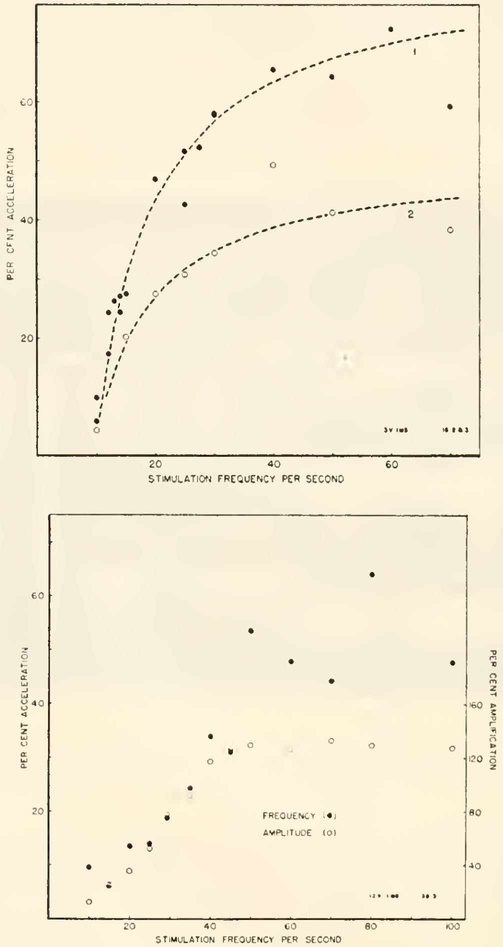


FIGURE 9. Cardio-accelerator; stimulus-response curves. A. ●, cardio-accelerator 1; ○, cardio-accelerator 2. Plot from individual of Figure 8A. B. Cardio-accelerator 2; the response curves are similar in shape for both frequency and amplitude. Plot of individual of Figure 8B.

The stimulus-response curves were usually hyperbolic (Fig. 9A), but in several instances a roughly sigmoid curve (Fig. 9B) was obtained. It was found impossible to obtain averages of several runs, for the curves varied with time and the individual.

Facilitation of the accelerating mechanism was much slower than of the inhibitor mechanism. It involved a gradual increase in both frequency and amplitude, though the latter effect was usually the more apparent. The slope of this curve, and consequently the duration of facilitation at the maximum response, was related to the frequency of stimulation. In some preparations it was not unusual to find that at lower frequencies (40 per second) facilitation was too rapid to be noted by the amplitude of the heart beat (Fig. 8). In fact, often the first few beats af a period of stimulation were of super-amplitude, caused apparently by the sudden high degree of excitation applied to the pacemaker which destroyed momentarily the equilibrium presumed necessary for a rhythmic beat. This could also be considered a period of facilitation if it is assumed that a slightly higher level of excitation is necessary to restore regularity at increased rate and amplitude. In some cases where the frequency of stimulation was fairly low (25 per second) there was a slow linear increase in amplitude after the first period of rapid facilitation.

The effects following the release of stimulation can be grouped into three classes. 1) There was an exponential decrease in rate and amplitude, bginning immediately or soon after the release of stimulation. Usually the duration of this was not over 10 seconds, though the final amplitude after stimulation may remain slightly greater than that before stimulation. 2) In three cases the exponential decrease fell to a level of subnormal amplitude and frequency which was followed by a gradual return to the normal beat. In the case of amplitude, this may last for nearly a minute. This effect was especially noted at high frequencies, when low frequencies yielded effect 1, and was to some extent dependent upon the duration of stimulation. 3) In two cases the stoppage of stimulation had very slight or an increased effect. The latter case must have been caused by after-discharge in the nerve. In the former, there was a sudden dip in amplitude upon the release of the stimulus followed by a slow return to normal amplitude lasting well over a minute. The rate returned to normal more rapidly. This is similar to lasting after-effects noted by Smith (1947) in the crab.

## Discussion

The System I fibers in the dorsal nerve are probably inhibitory. Their number corresponds well with the number of thresholds, and they make intimate connection with the large cell bodies and neuropiles within the ganglion. The homologous fiber in the stomatopods is inhibitory (Alexandrowicz, 1934). No evidence was obtained concerning the function of System II.

The several thresholds are probably best explained by assuming that new cardioregulator fibers were recruited with increasing stimulus strength. The stepwise increase in inhibition, as mentioned above, can thus be correlated with the limited number of fibers observed (2–4). Likewise the all-or-none type of response at each threshold is as expected if a limited number of fibers of different thresholds were stimulated with a continuously increasing stimulus strength.

Assuming the different thresholds indicate new fibers stimulated, both temporal and spatial summation are clearly shown, especially in the inhibitor. There, temporal summation is slightly more efficient than spatial. Both, however, follow essentially the same "exponential" stimulus-response curve.

Though the stimulus-response curves are quite different from a mathematical viewpoint, they probably do not indicate radically different physiological processes. As shown by Rosenblueth and Rioch (1933) in discussing the response curves of mammalian neuro-effector junctions, the change from an apparent hyperbola to a sigmoid need not indicate a change in the process. Rather, the relative rate of recovery of a preparation following a single nerve impulse, or, as they assumed the rate of destruction of a mediator, in relation to the frequency of stimulation could determine whether a sigmoid or a hyperbola would be obtained. Thus the sigmoid frequency-acceleration curve would indicate that the recovery following each impulse was more rapid in this preparation than in one which produced a hyperbolic curve. The inhibitor stimulus-response curve could also fit here, in which case it would correspond to the lower portion of a sigmoid. Complete inhibition would generally be reached before the flexion point. The short after-effect of inhibition as compared with acceleration is evidence for the required rapid recovery.

These curves, however, do not indicate whether the process is via the release of a chemical mediator as Rosenblueth and Rioch felt. It seems possible that an electrotonic potential, or some other effect, built up by a series of pulses could be the inhibiting or accelerating stimulus.

In any case, if an inhibiting mediator is present, it is probably not that noted by Parrot (1941) in the blood stream after inhibitory stimulation, which caused activity in the intestine, for this was obviously not rapidly destroyed.

The alternating rhythm deserves some mention. In the normal heart beat, a burst of nervous activity is followed by a silent period and then another burst (unpublished observation). The continuity of this regular action is assumed to depend on a balance between activity and recovery of all the cells of the ganglion. Under some conditions the excitability states of the ganglion cells get out of phase. A burst of activity may be just great enough to prevent all the cells from recovering completely by the time the next burst is initiated by the most rapidly recovering cell. Therefore, the second burst is smaller, some of the cells "fire" fewer times than before, and the following recovery period is shorter, for the interval seems dependent upon the activity during the burst. In the next burst, the number of cells completely recovered is greater. Therefore, there is a large burst that is followed by a smaller, and so on. If the excitability of the out-of-phase cells can be either raised or lowered, a regular beat should be re-established. As a corollary, if the excitabilities of some of the cells of a regularly beating heart are either raised or lowered more than of other cells, throwing them out of phase, the alternating beat should be established. Both of the above phenomena were observed upon stimulation of the extrinsic nerves. Usually the accelerators stopped the alternating beat, though in some instances a temporary alternation was begun in an otherwise regular beat. The inhibitors generally brought about the alternation, though at times they also served to stabilize. If the above is correct, we may, therefore, conclude that the extrinsic nerves appear to affect the excitabilities of the individual ganglion cells.

I wish to acknowledge the aid of Dr. John H. Welsh in formulating this problem and the cooperation of Dr. L. Hutchins and Dr. W. Sutcliffe at the Bermuda Station. I also wish to express appreciation to Dr. R. I. Smith and Dr. T. H. Bullock for criticizing the manuscript.

## SUMMARY

The morphology and aspects of the physiology of the cardiac ganglion and the extrinsic cardiac nerves of *Panulirus argus* have been studied.

1. The extrinsic nerves consist of two pairs of cardio-accelerators and one pair of cardio-inhibitors which arise in the anterior portion of the thoracic ganglion. These run dorsally and laterally to join at the pericardial plexus where fibers from each come together to form the dorsal nerve. This runs to the cardiac ganglion. The thick fibers (2–4) of the dorsal nerve which ramify over the large cells of the ganglion are probably inhibitors. The identification of the accelerator fibers remains unclear.

2. Upon electrical stimulation of the extrinsic nerves, two or three levels of response were obtained with increasing pulse duration and voltage. This was probably because of an increase in the number of fibers stimulated.

3. Complete inhibition was usually obtained by frequencies of 35, 50 or 90 stimuli per second, depending upon the level of response of the heart. Usually no inhibition was obtained below a frequency of 15 per second. Between the minimum and maximum there was a gradation of both frequency and amplitude of heart beat. The stimulus-response curves approximated an exponential curve. Facilitation and an after-effect were present but of short duration.

4. The maximum increase in frequency and amplitude of the heart beat was usually obtained by stimulation of the accelerators at 60 per second or more. No response was ordinarily obtained below 5–10 stimuli per second. The stimulus-response curve was usually hyperbolic, though in some cases it was sigmoid. The actions of the two accelerators appear to be identical. Facilitation and after-effect have a much longer time constant than in the case of the inhibitor.

5. From a consideration of the participation of individual cells in the cardiac ganglion discharge for each beat, it is probable that the cardio-inhibitor and accelerators depress or enhance the excitabilities of the ganglion cells.

## LITERATURE CITED

ALEXANDROWICZ, J. S., 1932. The innervation of the heart of the Crustacea. I. Decapoda. *Quart. J. Micro. Sci.*, **75**: 182–250.

ALEXANDROWICZ, J. S., 1934. The innervation of the heart of the Crustacea. II. Stomatopoda. *Quart. J. Micro. Sci.*, **76**: 511–549.

CARLSON, A. J., 1905. The nature of cardiac inhibition with special reference to the heart of *Limulus*. *Amer. J. Physiol.*, **13**: 217–240.

CARLSON, A. J., 1905–06. Comparative physiology of the invertebrate heart. IV. The physiology of the cardiac nerves of the arthropods. *Amer. J. Physiol.*, **15**: 127–235.

GARREY, W. E., 1942. An analysis of the action of acetylcholine on the cardiac ganglion of *Limulus polyphemus*. *Amer. J. Physiol.*, **136**: 182–193.

HEATH, J. P., 1941. The nervous system of the kelp crab, *Pugettia producta*. *J. Morph.*, **69**: 481–498.

HEINBECKER, P., 1933. The heart and median cardiac nerve of *Limulus polyphemus*. *Amer. J. Physiol.*, **103**: 104–120.

HEINBECKER, P., 1936. The potential analysis of a pacemaker mechanism in *Limulus polyphemus*. *Amer. J. Physiol.*, **117**: 686–700.

JASPER, H. H., AND A. M. MONNIER, 1933. Pseudo-chronaxies des systèmes neuromusculaires des crustacés dues a la réponse rhythmique du nerf. *C. R. Soc. Biol. Paris*, **112**: 233–236.

Krijgsman, B. J., 1952. Contractile and pacemaker mechanisms of the heart of arthropods. *Biol. Rev.,* **27**: 320–346.

Levin, A., 1927. Fatigue, retention of action current and recovery in crustacean nerve. *J. Physiol.,* **63**: 113–129.

Pantin, C. F. A., 1936. On the excitation of crustacean muscle. II. Neuromuscular facilitation. *J. Exp. Biol.,* **13**: 111–130.

Parrot, J. L., 1941. Recherches sur la transmission chimique de l'influx nerveux chez les crustacés. *C. R. Soc. Biol. Paris,* **135**: 929.

Prosser, C. L., 1943. Single unit analysis of the heart ganglion discharge in *Limulus. J. Cell. Comp. Physiol.,* **21**: 295–305.

Rosenblueth, A., and D. McK. Rioch, 1933. Temporal and spatial summation in autonomic systems. *Amer. J. Physiol.,* **106**: 365–380.

Smith, R. I., 1947. The action of electrical stimulation and of certain drugs on cardiac nerves of the crab, *Cancer irroratus. Biol. Bull.,* **93**: 72–88.

Welsh, J. H., 1939. Chemical mediation in crustaceans, II. The action of acetylcholine and adrenalin on the isolated heart of *Panulirus argus. Physiol. Zool.,* **12**: 231–237.

Welsh, J. H., and D. M. Maynard, 1951. Electrical activity of a simple ganglion. *Fed. Proc.,* **10**: 145.

Welsh, J. H., and R. Taub, 1950. Structure-activity relationships of acetylcholine and quaternary ammonium compounds. *J. Pharm. and Exp. Therapeut.,* **99**: 334–342.

Wiersma, C. A. G., and E. Novitski, 1942. The mechanism of the nervous regulation of the crayfish heart. *J. Exp. Biol.,* **19**: 255–265.

# RATE OF WATER PROPULSION IN MYTILUS CALIFORNIANUS AS A FUNCTION OF LATITUDE

K. PAMPAPATHI RAO[1]

*Department of Zoology, University of California, Los Angeles, California*

Although extensive studies have been made on the physiological effects of temperature change in poikilotherms, very seldom has this information been applied to a study of animal activity in relation to latitude. It is common knowledge that a lowering of temperature results in a reduction in metabolism in cold-blooded animals. Consequently, it is classically assumed that marine invertebrates inhabiting the colder waters of the higher latitudes have lower metabolic rates (Wimpenny, 1941).

However, there are many indications in the literature that species living in warmer and in colder latitudes are adapted in some degree to the temperature difference, not only in their tolerable limits but also in activity. For example, Takatsuki (1928) found that a species of oyster from Japan has a higher heart rate at any given temperature than another species from the tropical Pacific. Even within the same species, populations at higher latitudes are more active than those from lower at any temperature between the zones of heat depression and cold depression (Mayer, 1914 on *Aurelia;* Spärck, 1936 on several pelecypods including *Mytilus edulis;* Fox, 1936, 1938, 1939; Fox and Wingfield, 1937 and Wingfield, 1939 on polychaetes, crustacea and other groups). The result appears to be that at their natural temperatures, activity is nearly the same (Thorson, 1946, 1950 on pelecypod respiration and on the duration of larval life).

The review of Bullock (1951) covers the available information relating to temperature compensation in aquatic poikilotherms. Though the fact of the larger size of northern animals was known and the effect of increasing weight on the metabolism per unit weight of the organism was recognized, most of the earlier experimenters did not make quantitative evaluation of these factors in discussing their results. The present investigation is an attempt to understand the effect of temperature on an index of activity, the rate of water propulsion in representatives of a single species, *Mytilus californianus,* with special reference to the latitudinal distribution of the species and under weight controlled conditions. Though it is possible that the latitudinal differences in a species might be due to several factors, such as local nutritional conditions, oxygen and mineral content of the waters, for the present study temperature differences only are experimentally analyzed and correlated with the temperatures of the localities of origin of the animals.

It is a pleasure to acknowledge my debt to Dr. Theodore H. Bullock, not only for providing me with all the necessary facilities, but also for his enlightened interest in

---

[1] Work done during the tenure of a Smith-Mundt and Fulbright Fellowship of the U. S. State Department, awarded through the Institute of International Education, New York, for the year 1951–52.

every aspect of the investigation. To Dr. John L. Roberts for several courtesies extended to me, I am greatly indebted. Thanks are due to other members of the staff of the Zoology Department, for their generous attention to my several needs. Particular mention must be made of the kindness with which Dr. Emery F. Swan sent me mussels and detailed data from Friday Harbor, Washington.

## MATERIAL AND METHODS

*Mytilus californianus* Conrad, collected from Los Angeles (Lat. 34° 00′ N), Fort Ross (Lat. 38° 31′ N) and Friday Harbor (48° 27′ N), were used in the present investigation.[2] The mussels from the north were transported in moist air in insulated boxes and in both cases were transferred to aquaria of fresh sea water at a temperature chosen to approximate that at which they were collected not later than 48 hours after removal from their natural waters. To correspond with this treatment, local mussels were kept in insulated boxes after collection, for similar lengths of time, before being transferred to aquaria. Throughout the experimental period the mussels were kept at the supposedly natural temperatures which were 16°, 10° and 6.5° C., all ± 1.5° C., for Los Angeles, Fort Ross and Friday Harbor respectively. Sea water in the aquaria was continually aerated but was not running; it was changed once a week. This effectively eliminated the factor of excessive reduction in the salinity of sea water due to condensation at low temperatures. Over a week the salinity of the sea water in aquaria at 6.5° C. fell from 34.3% to 31.0% in extreme cases. The animals were not obtaining significant quantities of food.

The method described by Jørgensen (1943, 1949) was used for determination of the rate of water propulsion. This method depends on photoelectric estimation of the rate of clearing of suspensions of colloidal graphite. The same batch of graphite (Prodag, particle size 5–15 $\mu$) was used throughout the course of this investigation, although it was observed that the values obtained by using Aquadag (particle size 1–2.5 $\mu$) were not different. Pumping rate was not affected by the concentration of the suspension or its age within the limits used. Pumping rates as measured may not necessarily be equated with feeding rates, but the values obtained showed insignificant variation (see legend, Fig. 3) and correspond well with the values Jørgensen (1949) obtained on *Mytilus edulis* with flagellates and diatoms and which he interpreted as due to normal feeding with a "feeding mucus." The fact that the measured rates are constant for several individuals of any given weight from the same population, at any given temperature, permits of a comparative study. Each individual mussel was experimented upon from three to five times at each temperature, in vessels of 2 to 2.5 liters capacity. Ovigerous individuals were eliminated not only because they would effect the weight-specific rates but also because they were erratic in their activity.[3]

[2] All the mussels were collected from about 1½ to 2 feet above the 'O' tide level.

[3] Since writing the above account, data (to be published later) have been collected, which show that mussels from the low inter-tidal have a faster pumping rate compared to those 4 to 6 feet higher up on the same piling or rock surface. Further, a tidal-rhythmicity of the rate of water propulsion (which persists for over four weeks in the laboratory) in *Mytilus californianus* has been discovered. These two factors account for some of the variation reported above.

## Results

### *Shell-weight as a function of latitude*

While readings of weight of soft parts were being taken, it was noticed that the mussels from northern latitudes had relatively smaller percentage of the total weight as soft parts. Within the weight range recorded, shell weight is directly proportional to the wet weight of soft parts at any given latitude (Fig. 1A). The slopes of the lines show a consistent increase with latitude. When the shell weight is expressed as a percentage of total weight and is plotted against latitude (Fig. 1B), there is an initial rapid increase in the percentage of shell weight, which flattens toward the higher latitudes. But, if the same percentage values of shell weight are plotted against mean annual temperature of the given locality (Fig. 1C), there is a close relation between decrease in temperature and increase in percentage of shell weight. Factors like duration of exposure, crowding and nature of the surface might influence this ratio at local areas. Thus Fox and Coe (1943) obtained a

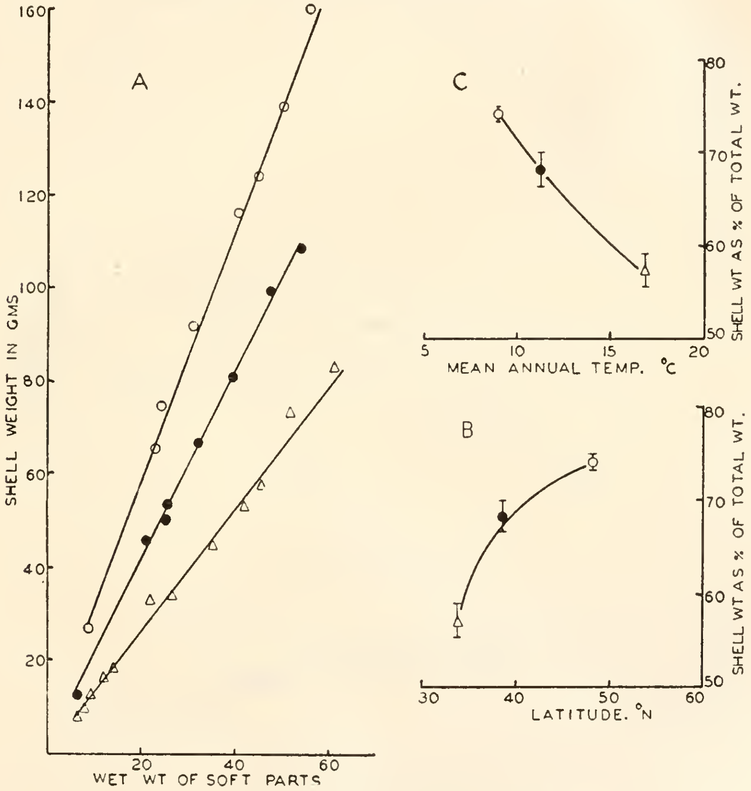


FIGURE 1. Weight of shell as a function of latitude in *Mytilus californianus.* △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Mean annual temperature data from Scripps Institution of Oceanography, La Jolla.

higher percentage of shell weight for mussels cultured under water without exposure, at La Jolla. These results are exceptions to the common generalization that animals of cold northern latitudes tend to have thinner and more fragile shells (Wimpenny, 1941).

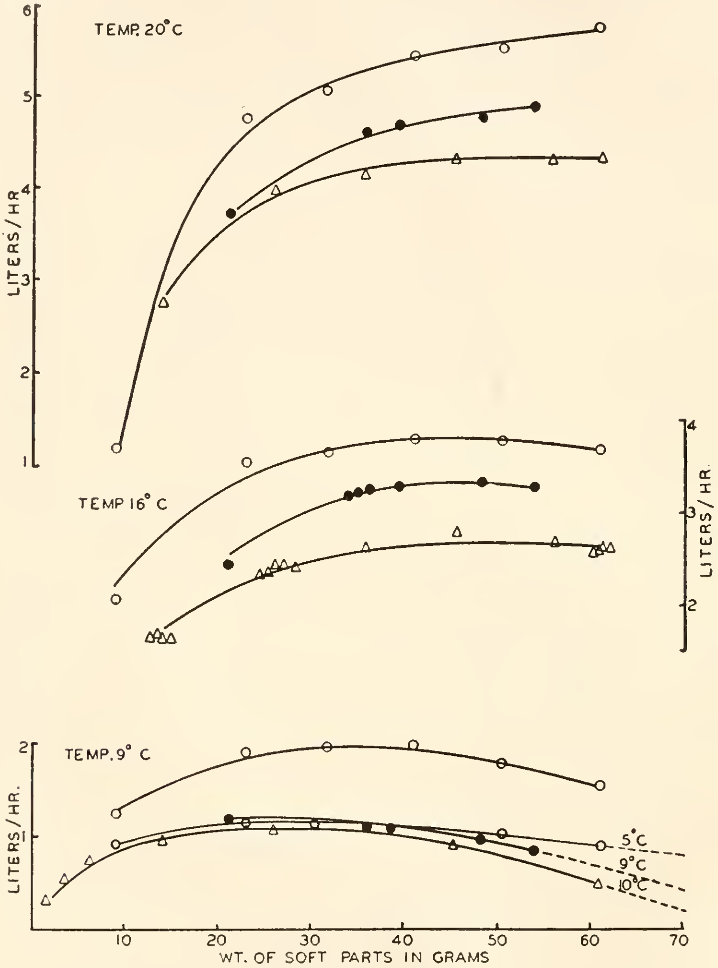


Figure 2. Absolute rate of pumping as a function of weight at different temperatures in *Mytilus californianus* from different latitudes. △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Each point represents the average for five to twenty-five readings. Each weight group consisted of three to five mussels. See Figure 3 for statement of variation.

### *Rate of water propulsion as a function of latitude*

A comparison of the effect of temperature on the absolute rate of pumping in animals of increasing weight, from different latitudes (Fig. 2), indicates that at any given temperature animals of equal weight from higher latitudes have a higher total pumping rate than those from more southerly latitudes.

At minimal temperatures (bottom, three curves) the absolute rate of pumping increases with increasing weight, until a certain point and then declines with increasing weight. This decline in absolute pumping rate with increasing weight is slower in higher latitudes. The fact that at their local minimal temperatures the northern animals can grow larger and yet remain as active as a much smaller animal from a southern locality at its own minimal temperature, correlates well with the known larger size of animals from higher latitudes.

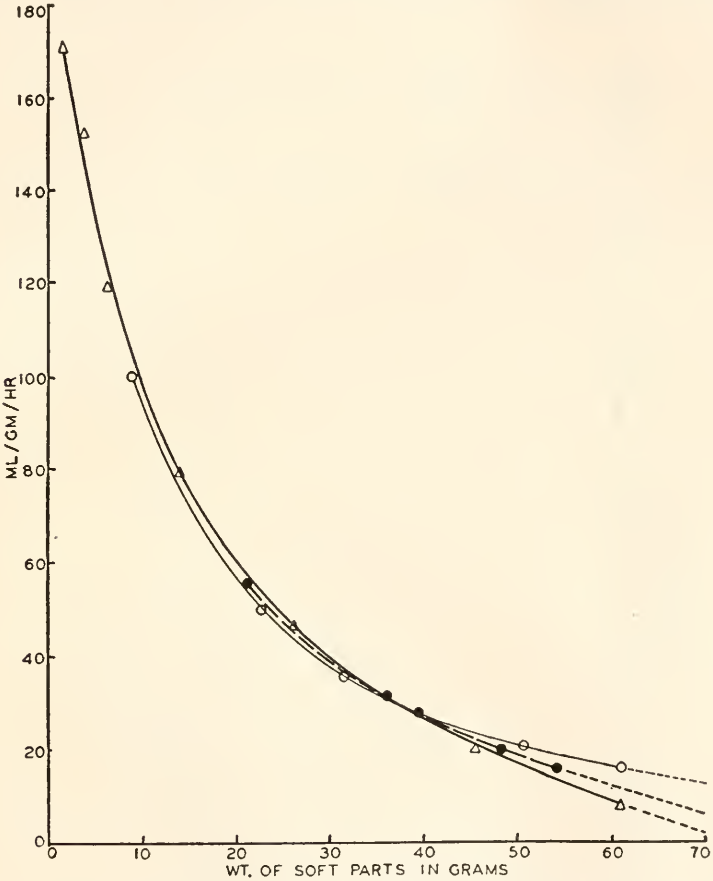


FIGURE 3. Weight-specific rate of pumping of *Mytilus californianus* from different latitudes at approximately minimal temperatures at these latitudes. △ Los Angeles at 10° C.; ● Fort Ross at 9° C.; ○ Friday Harbor at 5° C. Variation of rate in either direction not greater than $\frac{1}{2}\sqrt{n}$, where $n$ is ml./gm./hr.

The weight-specific rate of pumping of the mussels from different latitudes appear identical at the minimal temperatures encountered at the respective latitudes (Fig. 3) (according to best available sources—Scripps Institute of Oceanography and U. S. Coast and Geodetic Survey), except that the decline in rate of activity per gram in larger animals is slower in samples from the higher latitudes.

In the cold-adapted, higher latitude animals, the smaller individuals are the first to be affected adversely by high temperatures (Fig. 2, top curve).

Comparing the rate of pumping per unit weight in *Mytilus californianus* of similar weights from different latitudes (Fig. 4), it is evident that at any given temperature animals from the higher latitudes have a much higher rate per unit weight than those from lower latitudes. A 20 gm. animal from either Los Angeles or Fort Ross

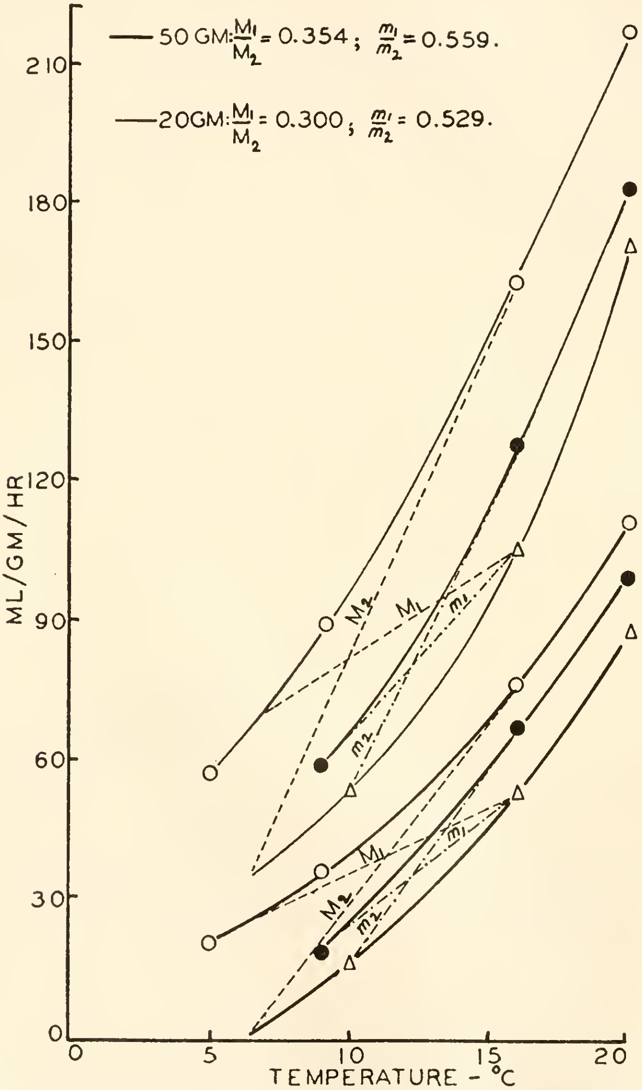


FIGURE 4. Rate of water propulsion as a function of latitude in *Mytilus californianus*. △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Variation of rate in either direction not greater than $\frac{1}{2}\sqrt{n}$, where $n$ is ml./gm./hr.

TABLE I

*$Q_{10}$ of rate of water propulsion of Mytilus californianus as a function of latitude and size*

| Latitude | $Q_{10}$(10–20° C.)<br>20 gm. | <br>50 gm. |
|---|---|---|
| Los Angeles:<br>34°00′ N | 3.2 | 5.44 |
| Fort Ross:<br>38°31′ N | 2.71 | 3.96 |
| Friday Harbor:<br>48°27′ N | 2.36 | 3.08 |

shows a higher rate per unit weight at all temperatures compared to an animal weighing 50 gm. from Friday Harbor.

*$Q_{10}$ of rate of water propulsion as a function of latitude and size*

An examination of the attached table, the results of which are plotted in Figure 5, reveals that over the range of 10–20° C. the $Q_{10}$ for the rate of water propulsion in *M. californianus* increases with increasing weight, while with increasing latitude

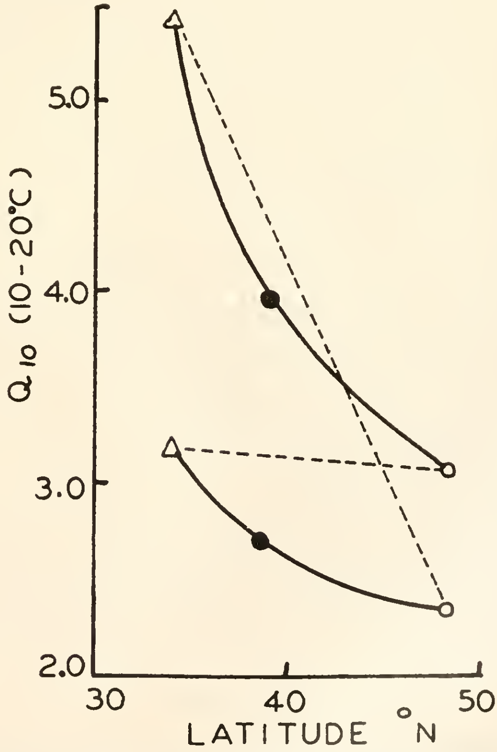


FIGURE 5. $Q_{10}$ as a function of latitude in two different weight groups of *Mytilus californianus*. Bottom curve, 20 gm. individuals; top curve, 50 gm. individuals.

it decreases. Roberts (1952) similarly shows that the $Q_{10}$ for oxygen consumption in *Pachygrapsus crassipes* increases with weight. The change in $Q_{10}$ over the whole range of temperature for each group indicates that the phenomenon reported here is real and is physiologic. Hitherto, it has generally been assumed, chiefly on the basis of the data of Edwards (1946) on insects, that the smaller individuals show greater response to temperature change. A closer examination and analysis of the same data reveal that in *Melanotus* the larger individuals have a slightly higher $Q_{10}$ at all temperatures. Especially marked is the difference at the lower temperatures recorded. The small individuals are not more sensitive to temperature change but have a higher rate at any given temperature, just as in the present case.

This relation between weight and $Q_{10}$ is obviously of the greatest consequence in comparing different weight groups and in the ecology and distribution of poikilotherms.

### *Temperature compensation coefficient*

A comparison of the rates of pumping of mussels of the same weight from different latitudes reveals that *M. californianus* from Friday Harbor has the same rate of pumping at 6.5° C. as the one from Fort Ross at 10° C., while animals from Los Angeles show the same rate at about 12° C. In comparing the degree to which groups of individuals from the same local population acclimatize to high or low temperatures in the laboratory, Roberts (1952) has proposed the ratio between the two slopes, $M_1$ and $M_2$, as a measure of the degree to which acclimatization has been achieved relative to the temperature change and has called it the acclimatization coefficient. $M_1$ is the slope of the line drawn between the highest temperature rate in the warm-adapted group and the lowest temperature rate in the cold-adapted, while $M_2$ is just the reverse (Fig. 4: $M_1$ and $M_2$; $m_1$ and $m_2$). This ratio is less than 1 if there is any acclimatization and approaches zero as the degree of acclimatization approaches perfection.

The same procedure may be applied for comparing rates of activity relative to latitudinal difference (assuming this to be chiefly a temperature difference), or, which is the same thing, to express the degree to which animals belonging to the same species located at different latitudes compensate for the temperature change. This may be called the *temperature compensation coefficient.* It cannot be called the acclimatization coefficient because we are dealing with separate populations which may have different genotypes and the term acclimatization should perhaps be reserved for purely phenotypic adaptation.

Interpreting Figure 4 in this light, it is seen that in general smaller animals have lower values for the coefficient, and that the temperature compensation coefficient is lower between Friday Harbor and Los Angeles than between Fort Ross and Los Angeles. In other words smaller animals compensate better than larger animals and more northern mussels show greater compensation to temperature change than those from lower latitudes.

## DISCUSSION

That the shell weight is a function of latitude and consequently of the mean annual temperature, is of interest, inasmuch as this morphological feature can be used to determine the physiological nature of a population. The consistency and small variance of the shell weight also permit its use as an indicator of the temperature of the environment. The relation with the environment may be as close as for the familiar "rules" for vertebrate coloration and proportions.

It is shown that the absolute rate of water propulsion (ml./hr./individual) at minimal habitat temperatures begins to fall after a certain weight is reached, and that this decline is slower in higher latitudes than in lower. The weight-specific rate (ml./hr./gm.), of course, falls with growth and the correlation between the larger size of the northern forms and their relatively slower decrease in activity per gram, plotted against size is similarly significant. Though this does not necessarily imply that the maximal weight to which animals can grow in a given locality can be computed with accuracy by an extrapolation of the available data, still it does show that small differences in temperature are of great significance and that a physiological basis can be found for a well known generalization in ecological geography. This phenomenon of smaller size-dependence of activity in higher latitudes emphasizes a need for obtaining geographically and temporally closely spaced temperature records in inshore waters. The work of Hubbs (1948, 1952) in this direction is noteworthy and is a timely beginning.

In this connection special interest is attached to populations of offshore, deeper water individuals of the same species, particularly of sessile forms like *Mytilus* which have widely dispersed gametes and larvae. If the physiological differences found in populations widely separated latitudinally are based on phenotypic acclimatization, it is to be expected that the same differences will be found between intertidal populations and those at 20 to 30 meters depth, where the temperature is several degrees lower. Adequate material for this comparison has not yet been studied but is available.

The importance of taking weight into consideration when comparing individuals or groups for their activity, especially at various temperatures, is shown not only by the fact that at all temperatures smaller individuals have higher rates as is well known, but by the more significant fact that the $Q_{10}$ as well as the temperature compensation coefficient are lower in smaller individuals. An examination of the data of Fox *et al.* (1936, 1939), already referred to, suggests that almost all the cases in which, at a given temperature, a *higher* rate of activity is reported from *lower* latitudes, are the result of using significantly smaller animals from the lower latitudes. The latter were, in these particular cases, from 2.3 to 45 per cent of the weight of the higher latitudinal animals with which they were compared. Of course the same factor acts to increase the significance of those cases reported where the forms from lower latitude have a *lower* rate at any given temperature, as the authors recognized.

In Figure 5, it may be noticed that the difference in $Q_{10}$ value between a small animal from the north and a larger animal from the south is much greater than that between a small animal from the lower latitudes and a larger animal from the higher latitudes. This means that an organism moving from the northern latitudes toward the lower as it grows must make a tremendously great physiological adjustment as compared to one moving from lower latitudes to the higher. Further, it was observed earlier that amongst the higher latitudinal animals, individuals which are smallest are the first to be affected adversely by raising temperatures. These two facts make it probable that the center of distribution in a species like *Mytilus californianus* lies in the lower latitudes.

The use of the temperature compensation coefficient as a quantitative measure of regulation makes possible analysis and comparison of otherwise not easily comparable physiological phenomena. Shifting an animal from its normal temperatures evokes, in organisms like the mussel, a compensatory response. The degree to which an organism responds and compensates may be related to the degree to which it is removed from its norm. As observed earlier, mussels from more northern latitudes show greater compensation to temperature change than those from intermediate latitudes, not only absolutely but relatively to the temperature difference. Instances which may be parallel are found in human metabolism. Thus, the net efficiency of excess metabolism (over the basal), while walking on level is 20 per cent whereas the net efficiency of excess metabolism, while walking on an 11.4 per cent grade, is 52 per cent (Bazett, 1949). Rothstein and Adolph (1944) found that the amount of voluntary dehydration in acclimatized men is higher, the higher the rate of sweating. Under conditions causing men to sweat less than 400 gms./hr., 95 per cent of the water lost was replaced, whereas men working on the desert and sweating 1000 gms./hr. replaced only 50 per cent of their loss. Gross (1952) reports that regulation against osmotic stress in crabs is greater the greater the stress. The same seems to be true in the regulation of basal metabolism by precise control of heat loss in arctic as compared with tropical mammals and birds (Scholander, *et al.*, 1950). Out of this seems to evolve a principle: the relative compensation for environmental stress increases with increasing departure from a norm, within limits.

## SUMMARY

1. Shell weight relative to the weight of soft parts is a constant in any given population of *Mytilus californianus*, but it increases with increasing latitude.

2. The absolute rate as well as weight-specific rate of pumping is greater at any temperature in mussels from higher latitudes than in those of the same weight from lower latitudes.

3. The rate of decline, in the absolute as well as weight-specific rate of pumping, with increasing size, is shown to be slower in higher latitudes than in the lower. This perhaps affords a physiological basis for the larger size of the more northern forms.

4. The $Q_{10}$ of the rate of pumping, between 10 and 20° C., is higher in larger individuals of any given population. But the $Q_{10}$ of individuals of the same weight at different latitudes, shows a decrease with increasing latitude.

5. It is suggested that the center of dispersal of species like *Mytilus californianus* is in the lower latitudes. This suggestion stems from the fact that the difference in $Q_{10}$ values between smaller individuals of lower latitudes and larger individuals of higher latitudes is negligibly small, while the opposite comparison results in a large difference, requiring a great physiological adjustment.

6. A comparison of relative degrees of compensation to temperature at different latitudes is made possible by a proposed temperature compensation coefficient. If the phenomenon under study is a phenotypic change in the lifetime of individuals, this becomes a measure of the degree of acclimatization (Roberts, 1952). This coefficient shows a greater degree of compensation for temperature in more northern samples and in smaller individuals.

7. Such a comparison, and several other instances from earlier work, seem to suggest that the relative compensation to change increases with increasing change.

## LITERATURE CITED

Anonymous, 1948. Surface water temperatures at Coast and Geodetic Survey tide stations, Pacific Ocean, U. S. Coast and Geodetic Survey, TW-2, revised 1948, 47 pp.

Bazett, H. C., 1949. The regulation of body temperatures. Chapter 4, in Physiology of Heat Regulation, ed. L. H. Newburgh. W. B. Saunders Co., Philadelphia.

Bullock, T. H., 1951. To what degree are aquatic cold-bloods independent of temperature? Unpublished MS read at Symposium on Factors in Distribution of Intertidal Organisms, Western Society of Naturalists Meeting, Claremont, Calif.

Edwards, G. A., 1946. The influence of temperature upon the oxygen consumption of several arthropods. *J. Cell. Comp. Physiol.*, **27**: 53–64.

Fox, D. L., and W. R. Coe, 1943. Biology of the california sea-mussel (*Mytilus californianus*). II. *J. Exp. Zool.*, **93**: 205–250.

Fox, H. M., 1936. The activity and metabolism of poikilothermal animals in different latitudes. I. *Proc. Zool. Soc. Lond.*, **106**: 945–955.

Fox, H. M., 1938. The activity and metabolism of poikilothermal animals in different latitudes. III. *Proc. Zool. Soc. Lond.*, **108**: 501–505.

Fox, H. M., 1939. The activity and metabolism of poikilothermal animals in different latitudes. V. *Proc. Zool. Soc. Lond.*, **109**: 141–156.

Fox, H. M., and C. A. Wingfield, 1937. The activity and metabolism of poikilothermal animals in different latitudes. II. *Proc. Zool. Soc. Lond.*, **107**: 275–282.

Gross, W. J., 1952. Osmotic regulation in selected invertebrates. Ph.D. dissertation, Univ. of California, Los Angeles.

Hubbs, C. L., 1948. Changes in the fish-fauna of Western North America correlated with changes in ocean temperatures. *J. Mar. Res.*, **7**: 459–482.

Hubbs, C. L., 1952. Personal communication.

Jørgensen, C. B., 1943. On the water transport through the gills of bivalves. *Acta Physiol. Scand.*, **5**: 297–304.

Jørgensen, C. B., 1949. The rate of feeding by *Mytilus* in different kinds of suspension *J. Mar. Biol. Assoc.*, **28**: 333-344.

Mayer, A. G., 1914. The effects of temperature upon tropical marine animals. *Papers from Tortugas Lab., Carnegie Inst.*, **6**: 3–24.

Roberts, J. L., 1952. Studies on acclimatization of respiration to temperature in the lined shore crab, *Pachygrapsus crassipes* Randall. Ph.D. dissertation, Univ. of California, Los Angeles.

Rothstein, A., and E. F. Adolph, 1944. Interim Report No. 16 to C. M. R. Quoted in Chapter 5, Physiology of Heat Regulation, ed., L. H. Newburgh. W. B. Saunders Co., Philadelphia.

Scholander, P. F., Raymond Hock, Vladimir Walters, Fred Johnson and Laurence Irving, 1950. Heat regulation in some arctic and tropical mammals and birds. *Biol. Bull.*, **99**: 237–258.

Spärck, R., 1936. On the relation betwen metabolism and temperature in some marine lamellibranches [sic], and its zoogeographical significance. *K. Danske Vidensk Selsk., Biol. Medd.*, **13** (5): 1–27.

Takatsuki, S., 1928. Records of oceanographic works in Japan, **1**: 102. Quoted in Wingfield, 1939.

Thorson, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Komm. Danmarks Fiskeri-og Havundersøgelser, Serie: Plankton,* **4**: 1–523.

Thorson, G., 1950. Reproduction and larval ecology of marine bottom invertebrates. *Biol. Rev.*, **25**: 1–45.

Wimpenny, R. S., 1941. Organic polarity: some ecological and physiological aspects. *Quart. Rev. Biol.*, **16**: 389–425.

Wingfield, C. A., 1939. The activity and metabolism of poikilothermal animals in different latitudes. IV. *Proc. Zool. Soc. Lond.*, **109**: 103–108.

# REPRODUCTION IN PHORONOPSIS VIRIDIS. THE ANNUAL CYCLE IN THE GONADS, MATURATION AND FERTILIZATION OF THE OVUM

JOAN C. RATTENBURY

*Department of Zoology, McGill University,*[1] *Montreal, Canada*

The question of the development, maturation and fertilization of the gametes is one of the many aspects of the biology of the Phoronidea on which available information is very incomplete. There are two accounts of gonad development (Ikeda, 1903; de Selys Longchamps, 1907), but there have been no previous studies of the maturation and fertilization of the egg. A related problem concerns the possibility of protandry in species other than the three known to be hermaphrodite. In most cases studies have not been sufficiently detailed to allow for a decision between protandry and dioecism.

The following account is concerned with an apparently dioecious phoronid, *Phoronopsis viridis*. It deals with the cyclic changes in the gonads, the maturation and fertilization of the egg, and includes some observations, made incidental to the main body of work, which bear upon the question of protandry in this group.

## METHODS

*Phoronopsis viridis* was collected from a tidal mud flat, Elkhorn Slough, situated in Monterey County, California, for a period of over 12 months at intervals of approximately four weeks. Before examination individuals were removed from their tubes by breaking the latter with fine forceps. Specimens from each collection were preserved in Bouin's fixative. Individuals used in the study of the gonads and nephridia were sectioned using either the paraffin or freezing technique. Paraffin sections were stained with Harris' hematoxylin and counterstained with alcoholic eosin. Material to be cut as frozen sections was bulk stained in alum cochineal before embedding in gelatin.

Eggs were obtained from mature specimens of *Ph. viridis* by puncturing the body wall in the reproductive region and allowing the ova to fall out. Since these eggs were found to be already fertilized they were simply pipetted into bowls of fresh sea water and allowed to develop at approximately 13° C.

Ova to be sectioned were fixed in Carnoy's fluid, stained whole by the Feulgen technique and counterstained in fast green. They were placed in a drop of horse serum on a slide, and the slide supported above a 50:50 mixture of glacial acetic acid and full-strength formalin in a warm petri dish. Within a few minutes the horse serum coagulated, and a small block containing the ova could then be cut from the drop on the slide. This block was then dehydrated, cleared, using tertiary butyl

---

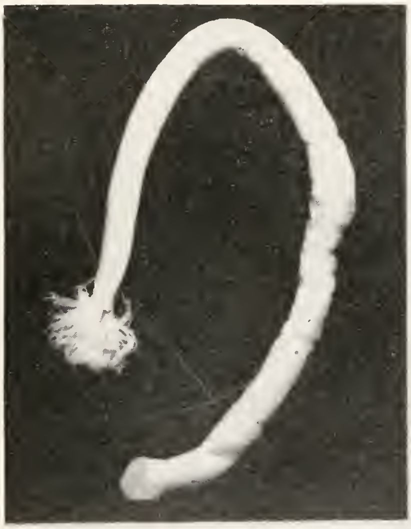
[1] This work was done while the author was a graduate student in the Department of Zoology at the University of California, Berkeley. The author would like to thank Dr. W. E. Berg, Dr. R. I. Smith and Dr. R. M. Eakin of that department for valuable encouragement and criticism during the progress of the work.

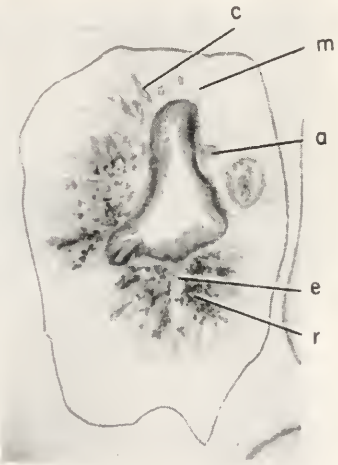
alcohol, and imbedded in paraffin. Sections were cut at 5 $\mu$ and drawings of the sections were made with the aid of a camera lucida.

Whole mounts of ova stained in phosphotungstic acid hemotoxylin or by the Feulgen technique were also studied.

## General Structure

*Phoronopsis viridis* was described by Hilton (1930) from specimens found at Moro Bay in southern California. The animals from Elkhorn Slough fit Hilton's description well with regard to those characteristics customarily used in the taxonomy of this group. These features are the general appearance, size, degree of spirality of the lophophore, number of tentacles and of longitudinal muscles and the position of the longitudinal nerve cord.


1



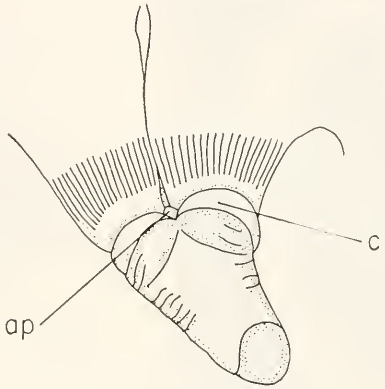
2

Figure 1. *Phoronopsis viridis.* The animal removed from the tube.

Figure 2. A cross-section of the body taken through the reproductive region. The reproductive tissue may be seen in the two anterior coelomic chambers. a—afferent vessel, c—capillary caecum, e—efferent vessel, m—mesentery, r—reproductive tissue.

*Phoronopsis viridis* is found in intertidal flats where the substratum is quite firm, consisting of a mixture of mud and sand. The animals inhabit tubes of a transparent secretion to which are cemented sand grains. The tubes, which are straight and occur vertically, vary in length from about 4 to 6 cm. and in diameter from 2 to 3.5 mm. The lower end tapers sharply in the last 3 or 4 mm. and is closed. The upper end of the tube is open and is usually flush with the surface of the mud. When the mud flat is covered with water the tentacles may project from the open end of the tube. The body is quite extensible and may extend 2 or 3 cm. beyond the end of the tube when the animal is not disturbed. The tube fits the animal tightly and it is difficult to remove the latter intact. The general appearance of the animal out of its tube is shown in Figure 1.

Both mouth and anus open at the base of the coiled lophophore which bears the tentacles. The anus is borne on a papilla which is situated between the two arms of the lophophore, and the paired nephridia open on either side of this papilla near its base (Fig. 3). Below the lophophore there is a fold in the body wall forming the collar, and below this again is the tubular body which contains the digestive tract, the longitudinal blood vessels and the gonad. There are four longitudinal mesenteries which divide this part of the body cavity into four coelomic spaces. As can be seen from Figure 2 these mesenteries are not evenly spaced along the circumference of the body and consequently the left and right posterior chambers are considerably smaller than are the two anterior chambers. The afferent blood vessel lies within the right posterior chamber and the efferent vessel within the left anterior chamber (Fig. 2). In the reproductive region of the body the efferent vessel gives rise to many short, blind branches, or capillary caeca (Fig. 2), and it is around these vessels that the gonads develop. The reproductive tissue is found only in the two anterior coelomic chambers, these areas being served by capillary caeca from the efferent vessel. The gonad is found in the aboral end of the body and may occupy from one- to two-thirds of the total length of the animal (Fig. 1). The body wall in this region is very thin and in season may become distended with gametes. The sexes are separate and may be easily distinguished during the breeding season, as the male gonad appears white and the female pink.



3

FIGURE 3. The oral end of the animal, posterior view. ap—anal papilla, c—collar.

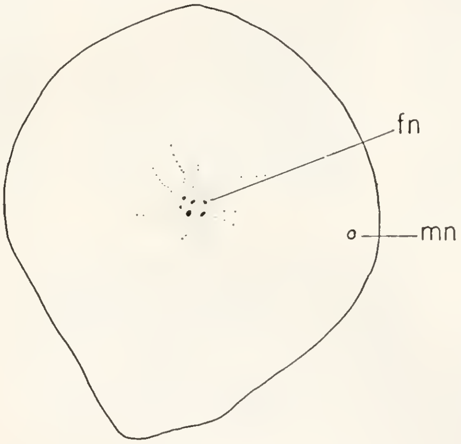
At times other than the breeding season a tissue known as the fat-body ("Fettkorper," Kowalevsky, 1867) or as vasoperitoneal tissue ("Vasoperitonealgewebe," Cori, 1939) is present around the capillary caeca. In life this tissue is jelly-like in consistency and transparent. Its structure, various inclusions and cycle of development are described below.

## Annual Cycle in the Gonads

The developmental sequence in the gonad of *Phoronopsis viridis* is very similar to that described for *Phoronis ijimai* and *Phoronis australis* by Ikeda (1903) and for *Phoronis psammophila* by de Selys Longchamps (1907).

*Female Gonad.* The breeding season for *Ph. viridis* lasts throughout most of March and April. In the case of the female, the gonad in late February contains large numbers of full-sized ova, each about 60 $\mu$ in diameter. At this time most of the ova still possess the large germinal vesicle. They may be packed very closely and each is covered by a thin, squamous epithelium, one cell thick. The remnants of the vasoperitoneal tissue take the form of small patches or strands of granular cytoplasm between the masses of ova.

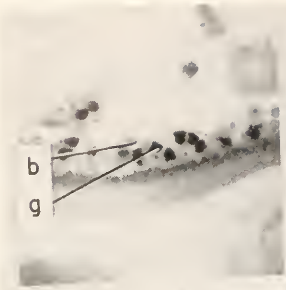
By about the middle of March many of the ova have become freed of the investing membrane. As this happens the germinal vesicle breaks down and the first maturation division begins. This division proceeds to first metaphase and stops (Fig. 4). The female nucleus remains at this stage until the egg is shed from the body. Once the ovum is freed of its investing epithelium it moves toward the periphery of the body and in the space between the outer surface of the gonad and the body wall the ripe ova collect. Occasionally an ovum will proceed to divide while still in the body cavity. Up to three or four such embryos have been found in one adult. Those found have always been blastulae. Similar embryos have been seen by de Selys Longchamps (1907) in *Phoronis mulleri* and *Phoronis sabatieri*.



4

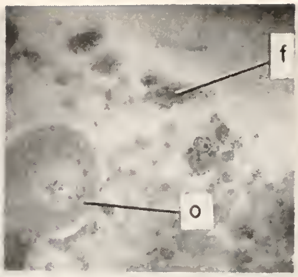
Figure 4. Camera lucida drawing of an ovum from the body cavity. The female nucleus is in metaphase. From a female collected in March. fn—female nucleus, mn—male nucleus.

Throughout the months of March and April large numbers of ova ripen and become free in the body cavity, where fertilization takes place. By the end of April most of the large ova have been spawned and there remain along some of the

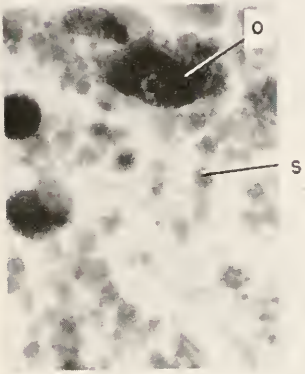


5

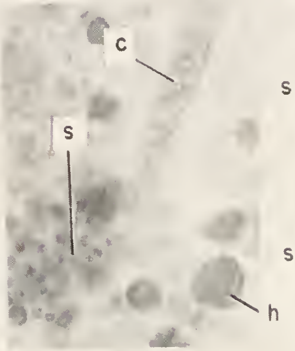
6



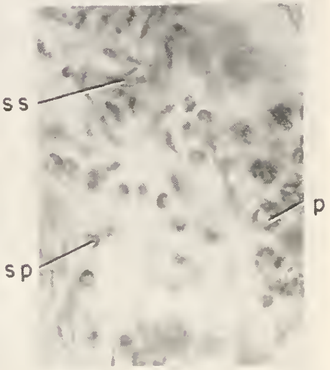
7



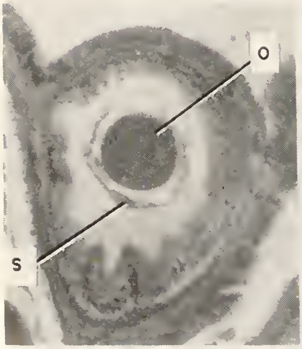
8



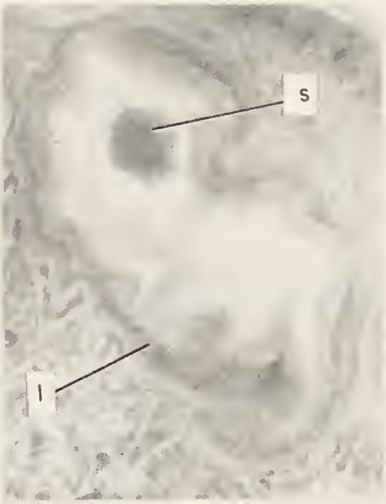
9



10



11



12

13

14

FIGURE 5. Opaque, non-staining granules, seen in a blood vessel. b—blood corpuscle, g—granule.

FIGURE 6. Dark green bodies, photographed from a smear of living tissue.

capillary caeca a considerable number of small ova, usually no more than 10 or 15 μ in diameter and frequently much smaller. These ova are presumed to be the forerunnners of next year's crop of eggs which began their development before this year's crop was shed.

At the end of April it is possible to examine some of the capillaries while they are entirely free of reproductive tissue. The walls of such vessels have been found to consist of a single layer of small, flat cells. These cells are said to be a part of the mesodermal splanchnic epithelium (Cori, 1890) and the capillaries, therefore, to be merely spaces between sheets of splanchnic peritoneum. Radiating from the capillaries are strands of tissue containing occasional nuclei, separating clear unstained spaces which are presumably filled with fluid in life and represent the spaces left by the discharge of the ripe ova.

In the body cavity of *Ph. viridis* and particularly in the reproductive region, there are a number of kinds of cells or bodies which may be found free in the coelomic fluid, or contained within the vasoperitoneal tissue. Such elements are present only in small numbers in and around the gonad at the breeding season, but may be found in this region in greater abundance at other times of the year. They have not been studied in sufficient detail to make possible an opinion of their functional role. There are, however, three types of bodies which have been seen repeatedly in almost all animals studied and these are described below.

The most obvious and frequently the most abundant of these elements is a fusiform body about 15 μ long. It stains a uniform pink with eosin, contains no apparent nucleus, and the surface is often marked with faint longitudinal lines (Fig. 7). Similar bodies have attracted the attention of many persons working with the Phoronidea and it has been suggested by Pixel (1912) that they are granules of nitrogenous waste material because of a positive reaction to the murexide test. As far as I know no other attempt has been made to determine their chemical nature.

In some, but not all of the animals studied there appeared groups of small, opaque granules (Fig. 5), which do not stain with either hematoxylin or eosin and which retain their color throughout fixation and dehydration. Their color under transmitted light is gold; they vary in size from 2 to 8 μ and have been found in the

---

FIGURE 7. Fusiform, eosinophilic body, seen in a female animal. f—fusiform body, o—ovum.

FIGURE 8. Non-granular, eosinophilic spheres in the vasoperitoneal tissue. o—ovum, s—eosinophilic sphere.

FIGURE 9. Coarsely granular, hematophilic body in the vasoperitoneal tissue. c—capillary caecum, h—hematophilic body, s—spermatocytes.

FIGURE 10. Section through the gonad of a male animal collected in February. p—primary spermatocyte, sp—spermatozoa, ss—secondary spermatocyte.

FIGURE 11. Section through the nephridial duct of a female animal collected in March. o—ovum, s—spermatozoa.

FIGURE 12. Section through the nephridial duct of a male animal collected in March. l—columnar lining of duct, s—spermatozoa.

FIGURE 13. Aceto-carmine squash preparation of the metaphase nucleus of an ovum in the body cavity. There are 13 pairs of chromosomes.

FIGURE 14. Aceto-carmine squash preparation of the male nucleus found in an ovum in the body cavity.

blood vessels as well as in the body cavity. Similar granules have been described from *Phoronis psammophila* by Cori (1939), who believed them to be inactive and degenerate amoebocytes.

At any time of the year some individuals contain minute dark green bodies in the coelomic cavity (Fig. 6). These bodies are non-motile, occur singly or in groups of two or more, and may perhaps be algal cells.

Following spawning, the development of next year's ova continues and many new ova appear. The development of new ova is most rapid at the proximal ends of the capillary vessels, on the efferent vessel and on the walls of the blood sinus surrounding the digestive tract. The germ cells are at first small, roughly spherical and contain a few darkly staining granules. They are separated from the lumina of the capillaries by a single layer of flat cells and are covered by a similar layer. In the case of the lateral vessel these epithelial cells lie outside the muscular elements in the wall of the vessel. The reproductive cells appear to be produced continuously, so that any given length of capillary wall will bear a series of ova of different ages, the oldest lying farthest from the parent vessel.

As the new generation of ova is beginning to develop, the vasoperitoneal tissue reappears. As early as May the clear spaces left by the discharged ova begin to fill with a finely granular cytoplasm containing many eosinophilic spheres of various sizes (Fig. 8). The cytoplasm and granules appear to be contained within very large cells separated from one another by membranous walls containing the nuclei. These cells appear to vary in length from about 25 μ to 75 μ and in diameter from about 6 μ to 14 μ. In general they are arranged around the blood vessels so that the long axes of the cells are at right angles to and radiate from the long axis of the capillary. There may be exceptions to this pattern, but since the capillary caeca pass in a number of directions it is difficult to determine by means of sections the relationships of all the cells of the vasoperitoneal tissue. The size and arrangement of these cells is similar to that described for *Phoronis psammophila* by Cori (1939). The developing ova become imbedded in the extensive peritoneal tissue and push into it as they increase in size.

There are two main types of inclusions found only in the cells of the vasoperitoneal tissue. The more abundant of these is the above-mentioned non-granular sphere, varying in diameter from less than 1 μ to about 8 μ (Fig. 8). These spheres stain with eosin and come to fill most of the substance of the vasoperitoneal tissue. The other type of inclusion is a coarsely granular, roughly spherical body varying in diameter from about 5 to 15 μ. This type of body stains with hematoxylin and appears in greatest abundance in the late summer and fall (Fig. 9).

In July all the ova are small and are found chiefly around the proximal ends of the capillaries, close to the digestive tract. By November the reproductive tissue has spread along the blood vessels, covering them for most of their length. At this time the larger ova are about 35 μ long and many new ones are being formed. By January the ova have increased in size, the larger ones being about 50 μ long and usually somewhat flask-shaped. Each ovum is still surrounded by a single layer of flattened cells. The germinal vesicle is large and contains a diffuse chromatin network as well as a large nucleolus in the shape of a curved disc. In February the gonad is packed with many large ova, 50 to 60 μ in diameter, and there are still many small ones along some of the capillaries. By this time the vasoperitoneal tissue has been almost eliminated; the inclusions found in this tissue in late summer and fall have disappeared and all that remains in the occasional spaces between ova are thin strands of tissue which probably represent the membranes of exhausted cells. It has been impossible to determine whether the developing ova penetrate into or between the cells of the vasoperitoneal tissue. Within the next month the ova ripen, are spawned, and the cycle of development of the gonads begins again.

*Male gonad.* Fully formed, active spermatozoa are found in male animals as early as December. The quantity of active male gametes increases throughout January, February and March. In March the aboral ends of male animals are usually a dense white due to the masses of spermatozoa inside. Spawning may take place in February, March and April.

In May, immediately after the end of the breeding season, there is apparently no reproductive tissue left. In contrast to the situation in the female, the tissue which in the male will give rise to next year's gametes does not appear until the breeding season is over and the present crop of spermatozoa shed. Later in May the vasoperitoneal tissue re-forms and the two types of inclusion described in the case of the female appear. At this time a few cells which are probably spermatocytes may be seen along some of the capillaries. By June the vasoperitoneal tissue is well formed and reproductive cells are abundant along the blood caeca. By August the quantity of reproductive tissue has increased considerably and throughout the fall months the proportion of generative tissue increases steadily as the extent of the vasoperitoneal tissue decreases.

The germ cells of the male arise in the walls of the blood vessels in the same manner as do those of the female. Spermatocytes can be distinguished readily in sections, but spermatogonia have never been satisfactorily identified. It seems probable that they resemble the primary spermatocytes. The latter are roughly spherical cells, about 3 $\mu$ in diameter, in which the nuclear material occupies most of the cell body. They are found immediately adjacent to the capillary walls, sometimes forming masses up to 20 cells deep. In some cases a single row of such cells along a capillary wall has been observed to be covered by a sheet of thin epithelial cells. No such epithelium has been seen in connection with larger masses of spermatocytes or their derivatives. The secondary spermatocytes are smaller, about 1 $\mu$ in diameter, and are typically found farther from the capillaries than the primary spermatocytes. The secondary spermatocyte consists almost entirely of nucleus and in some cases attempts were made to count the chromosomes. The highest number counted was 12. In December and throughout the breeding season the groups of spermatocytes become fringed with masses of fully formed spermatozoa. The latter appear first in pairs, often attached to one another at one end and for a part of their length (Fig. 10). Such pairs are usually distributed between the secondary spermatocytes and the single spermatozoa. During the breeding season clumps of spermatoza, aggregated so that the heads are together and the tails free, are frequently found in the body cavity of male individuals. When such clumps are placed in sea water they tend to disperse.

As in the female, the male gametes develop at the expense of the vasoperitoneal tissue. During the breeding season and immediately afterward the latter tissue is represented only by cytoplasmic strands radiating from the walls of the capillaries.

When spawning begins in the male there are many spermatocytes present, but by the end of the breeding season they have all disappeared and it is not clear whether they all develop to spermatozoa or whether some spermatocytes degenerate without reaching maturity. It is also possible that the small size of the spermatocytes may render them so inconspicuous in small numbers that under these conditions they appear to be absent. At any rate, a fairly careful study has failed to reveal any male reproductive tissue in the gonad immediately after the close of the breeding season.

It seems likely that both sexes spawn more than once during the breeding season. Sections of the gonad taken in March and April show areas depleted of gametes as well as areas filled with mature germ cells. The mid-body region of such animals is usually not filled with gametes and it seems probable that the eggs and spermatozoa from the depleted areas of the gonad have already been spawned. In the laboratory the animals usually spawn at night, emitting a slow stream of eggs or spermatozoa, but the quantity of material released under these conditions must certainly represent only a fraction of the total quantity produced.

## PROTANDRY

It has been suggested by a number of authors that some species of Phoronidea may be protandric, being first male and then female. It seems certain in the case of *Ph. viridis* that at any one breeding season an animal is either male or female. Moreover, from a study of the development of the female gonad it seems probable that a female at one breeding season will be a female at the next, as the ova for the following year are already present in small numbers. In the case of the male the evidence is inconclusive. The reproductive tissue disappears or else becomes exceedingly inconspicuous after spawning and once the season is over there is no way of recognizing a male except by the absence of ova.

If *Ph. viridis* were truly protandric, all individuals when they first reached sexual maturity would be males, and at some subsequent time would become females. If this were so it would be reasonable to expect that males might tend to be smaller than females. There is a considerable range of size among mature individuals of this species, but the length of life and rate of growth are unknown. Also it is well known that individuals may cast off the lophophore and tentacles when disturbed, and it is possible that constriction resulting in separation may occur in other parts of the body. Cases of fission of this type have been reported for *Phoronopsis albomaculata* (Gilchrist, 1907) and for *Phoronis ovalis* (Marcus, 1949). If this type of asexual reproduction does occur in *Ph. viridis*, then the length of the adult body may bear no direct relationship to the age of the animal. There has, however, been found no good evidence of asexual division in this species and hence measurements were made of the lengths of a number of male and female individuals to determine whether or not the sexes showed any significant difference in length.

These measurements were made throughout one breeding season (1949) on all animals removed intact from their tubes. A total of 169 animals was measured, of which 95 were males and 74 were females. The average length of males was 5.6 cm. and of females 6.75 cm. The standard deviation was 1.35 in the male group and 1.01 in the female group. The standard error of the difference between the means of male and female populations (d/d) was calculated according to the formula in Simpson and Rose (1939), and was found to equal 0.545. When this value is referred to the tables of "t" the probability is found to be 0.6 (p value).

From this it is apparent that there is no significant difference in length between male and female individuals. The males are not significantly smaller than the females, as might be expected if the animals were protandric. This analysis does not, of course, prove that *Ph. viridis* is not protandric; it merely indicates that on the basis of length of body there is no evidence for protandry.

## Spawning, Fertilization and Maturation of Ova

The gametes of *Ph. vididis* pass out of the body by way of the nephridia. There are two nephridia which open to the exterior on either side of the anal papilla. Each nephridium is roughly U-shaped; the longer arm of the U opens to the exterior by a small pore; the shorter arm terminates in a pair of long funnels. One funnel of each nephridium opens into the posterior coelom and one into the anterior coelom. The walls of the duct are lined with columnar epithelium bearing very long, fine cilia (Fig. 12). The cells lining the funnels are also columnar and ciliated, but are narrower and more densely packed. The nephridia extend through the collar region of the body and are about 0.5 mm. long.

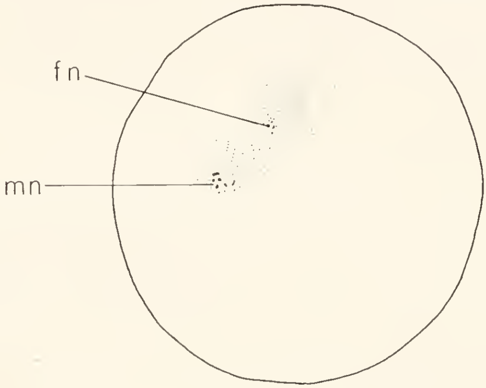
Examination of living animals at the time of breeding reveals that the contents of the reproductive region of the body are in constant motion as a result of contractions of the muscles of the body wall in the mid-body and reproductive regions. It seems probable that these movements are instrumental in getting the ova, once freed of investing membranes, up into the oral end of the body near the funnels of the nephridia. The average body length is about 5 or 6 cm.; the ova mature some 3 cm. from the nephridial funnels and must in some manner be propelled this distance before spawning can take place.

Sections of male animals, preserved in March, at the height of the breeding season, show large numbers of spermatozoa in the nephridium (Fig. 12). The spermatozoa occur in concentrated, roughly spherical masses which are found in both arms of the nephridium.

Sections of female animals preserved in March show both ova and spermatozoa in the lumen of the nephridium and in the body cavity (Fig. 11). The spermatozoa in the female nephridium may occur in dense masses and in some cases an ovum may be seen passing through a loose mass of spermatozoa (Fig. 11). Similar masses have been found in the mid-body region of a female collected in January. During the months of February, March and April spermatozoa may be found dispersed throughout the reproductive region of the female. No male tissue has ever been found in an otherwise female animal and it is probable that the spermatozoa which appear in the body of the female in January or February have come from a male animal. It seems likely that the spermatozoa enter the body of the female through the nephridium and fertilize the ova as they become free in the coelom. A male nucleus can usually be seen in ova which are free in the coelom and in which the female nucleus is in first metaphase (Fig. 4), but has not been found in ova which are still covered by the investing epithelium and in which the germinal vesicle remains intact. The male nucleus is very small and has been identified only by means of the Feulgen technique.

Once the ova has passed, by natural or by artificial means, from the body cavity into sea water the egg nucleus becomes active. Aceto-carmine squash preparations of ova from the body cavity reveal 13 pairs of chromosomes (2 n complement) on the metaphase plate (Fig. 13). The male nucleus may be seen in the preparations, although the chromosomes are not distinguishable (Fig. 14). Ten minutes after release into sea water the chromosomes of the egg nucleus have passed from metaphase to anaphase. At fifteen minutes the first polar body is forming. Thirty minutes after liberation the chromosomes are in metaphase of the second maturation division which takes place near the surface of the egg, usually very close to the first polar body. As the second polar body is forming the first usually divides.

During the first thirty minutes after liberation, while the two maturation divisions are taking place, the male pronucleus remains inactive. It may be found in a variety of positions within the egg, usually at some distance from the female pronucleus. Shortly after the formation of the second polar body the individual chromosomes of the male pronucleus become visible and an aster forms around them (Fig. 15). About forty-five minutes after liberation, both male and female pronuclei have moved to the center of the egg, forming a fusion nucleus of short duration. One hour after liberation this nucleus is in metaphase or anaphase of the first cleavage.



15

FIGURE 15. Camera lucida drawing of an egg fixed forty minutes after liberation from the body cavity. Asters are forming around both male and female pronuclei prior to the formation of a fusion nucleus. fn—female nucleus, mn—male nucleus.

As the polar bodies are being formed it becomes apparent that the egg is invested in a thin, closely fitting membrane which is now detectable by virtue of being lifted from the surface by the extrusion of the polar bodies.

## DISCUSSION

*Gonads.* From the preceding account it is clear that the yearly turnover in the gonads involves two cyclic processes in opposite phase. One of these is the cycle of development of the sex cells, beginning in the months of June and July and culminating in mature gametes in the following March and April. The other cycle is that of the vasoperitoneal tissue which reaches its ebb at the time of the breeding season and its peak in July and August in the male, or in September and October in the female. The vasoperitoneal tissue thus reaches its greatest development at a time when the gonads are at their smallest, and as the gonad increases in size the vasoperitoneal tissue becomes commensurately reduced. This relationship, together with the intimate contact which exists between the developing gametes and the vasoperitoneal tissue, has led other investigators (Ikeda, 1903; de Selys Longchamps, 1907) to assume that the substance of the vasoperitoneal tissue nourishes the growing germ cells and is therefore exhausted at the time of the ripening of the latter. Ikeda (1903) considers that the cells of the vasoperitoneal tissue act as follicle cells in relation to the gametes, and that the latter absorb nutrient from the vasoperitoneal cells. De Selys Longchamps (1907), however, holds that the spermatocytes and oocytes become free in the body cavity, pass into the cells of the vasoperitoneal tissue and there complete their development. Observations on *Ph. viridis* show that as the ova develop they are covered by a layer of epithelium which disappears, releasing the ova into the body cavity at the time at which the germinal vesicle breaks down. In the case of the male there is no detectable investing membrane and it is possible that the spermatocytes do undergo at least a part of their development in the body cavity. In either case, however, there is no evidence that the germ cells penetrate into the vasoperitoneal cells. It certainly seems possible that the vasoperitoneal tissue nourishes the germ cells in *Ph. viridis*, but the exact nature of the relationship between the two tissues is not clear.

After spawning there remain strands of tissue radiating from the blood caeca and between these strands are the spaces left by the discharge of the ripe gametes. It has been assumed that these strands are the remains of the vasoperitoneal tissue and, in the case of the female, also of the epithelium that once covered the ova. These spaces may be considered either as the now vacant intercellular spaces or, less probably, as the enlarged interiors of vasoperitoneal cells. Later in the year the radiating strands are not prominent and the vacant spaces fill up with new vasoperitoneal tissue. The relationship between the old strands and spaces and the new tissue is uncertain, although it appears that the new tissue gradually forms around the remains of the old. The fully formed vasoperitoneal cells appear to be constructed rather like watery sacs containing globules of various sizes, the nucleus being near the cell membrane. Ikeda (1903) states that in *Ph. australis* and *Ph. ijimai* the vasoperitoneal tissue arises by a proliferation of the peritoneal layer forming the walls of the blood vessels. Cori (1939) says that the situation is similar in *Ph. hippocrepia*. In *Ph. viridis* it has not been possible to determine the origin of this tissue.

Concerning the origin of the reproductive cells, Ikeda's (1903) account for *Ph. ijimai* and *Ph. australis* is in agreement with the findings for *Ph. viridis*. Although oogonia and spermatogonia have only been tentatively identified in *Ph. viridis*, it seems quite certain that they must arise from the peritoneal covering of the blood vessels as described by Ikeda.

*Protandry.* Apart from the work on the three species which have been shown to be hermaphrodite (*Ph. hippocrepia, Ph. ijimai* and *Ph. australis*) there is very little information concerning the question of protandric hermaphroditism versus dioecism in this group. De Selys Longchamps, working with *Phoronis psammophila,* found that most individuals contained either testis or ovary, but he obtained one specimen in which the testis surrounded a group of developing oöcytes. On the basis of this evidence *Ph. psammophila* has generally been considered as a protandric hermaphrodite. In several other cases species collected at only one time of the year have shown only ovaries or testes (*Phoronis vancouverensis* and *Phoronopsis harmeri,* Pixel 1912, and *Phoronis mulleri,* de Selys Longchamps 1902). Torrey (1901) found only testes in specimens of *Phoronis pacifica* collected in Humboldt Bay in June, whereas specimens from Puget Sound collected in another year (month not given) contained ova in the nephridia. He suggested that *Ph. pacifica* might be dioecious. Marcus (1949) reports that *Phoronis ovalis* from Brazil contains testes in May and oöcytes in July but it is not known whether or not this species is hermaphrodite.

Brooks and Cowles (1905) have made a more detailed analysis of the cyclic changes in the gonad in *Ph. architecta.* In this species there exists a situation somewhat similar to that described for *Ph. viridis. Ph. architecta* sheds its eggs freely into the water and at the breeding season individual animals contain either testes or ovary, but never both. Male animals possess a lophophoral organ and female animals do not. The males are mature from March to October and the females from May to October. Brooks and Cowles decided that this animal was probably protandric, that the males which were mature in March and April, before the onset of egg laying, had developed from eggs laid early in the previous year, that is, in May or June, and that these male individuals having spawned in March and April of their first year would become females by May. The males which matured in May and later were considered to have developed from eggs laid late in the previous breeding season which had not yet had time to pass through the male phase and become female. The lophophoral organs were found to contain spermatozoa and were considered to act as storage places while the animal changed from the male to the female state. Although spermatozoa were found in the body cavity of females, fertilization was considered to take place in the tentacular crown.

If this hypothesis be applied to *Ph. viridis* several difficulties arise. In the first place there is no lophophoral organ and therefore no storage place for the spermatozoa. In the second place the breeding season for *Ph. viridis* is much shorter; the eggs are shed over a period of only two months as compared with six in the case of *Ph. architecta.* This means that individuals of *Ph. viridis* conceived at the beginning of the egg-laying period could at most be only two instead of six months older than those developing from eggs laid at the end of this period, and such individuals are therefore less likely to have had time to pass through the male phase and become female. Finally it seems that the theory of Brooks and Cowles is, in the case of *Ph. viridis,* a rather roundabout way of explaining a phenomenon which could be a result of a situation in which there was no change of sex during the breeding season, but simply one in which the males matured earlier than the females which would ensure an abundance of spermatozoa when the ova became ripe. The fact that large masses of spermatozoa are found in the nephridia of males suggests that these spermatozoa are shed by the males, and the presence of similar masses in the nephridia and body cavity of females suggests that these masses are collected by the female. The method of this collection is an interesting problem which might be partially elucidated by a careful study of the currents of the branchial crown and lophophore. It is suggested that the spermatozoa are discharged in compact masses by male animals, some of whom will be no more than a centimeter from the nearest female. The masses of spermatozoa could then be drawn into the tentacular crown of the female by the currents created by the cilia on the tentacles and in some manner passed into nephridia. It is unknown whether the spermatozoa pass into the nephridium by active swimming movements or are swept in by the cilia on the nephridium. The presence of large, compact masses of spermatozoa in the female suggests that they may have been passed inward passively. Considering the probably normal excretory function of the nephridium and the size and position of the nephridiopore, spermatozoan entry is a difficult matter to explain satisfactorily.

Even assuming that the above course of events does take place, there remains the possibility of a change of sex from one year to the next, so that animals which are males one season will be female the following one. If this were so it might also be possible that the spermatozoa seen in the body cavity of the female were left over from the previous male phase and had not entered from the outside. This possibility seems somewhat unlikely since it would necessitate the maintenance of active spermatozoa for a period of eight months, would result in self-fertilization, and would not explain the presence of spermatozoa in the nephridia of both males and females, or the apparent lack of spermatozoa in the reproductive region of females from April to December.

On the basis of present information, therefore, it seems most probable that in *Ph. viridis* the sexes are separate, at least during any one breeding season, and that spermatozoa leave the male and enter the female via the nephridia. Since the evidence is by no means complete, the possibility of protandry cannot be ruled out, but it is at present considered unlikely.

*Fertilization.* Although spermatozoa have been seen in the body cavity of the female by a number of authors (Brooks and Cowles, 1905; de Selys Longchamps, 1907), they have not been previously described from the nephridium of the female and it has been generally assumed that fertilization took place outside the body, in the tentacular crown. Torrey (1901) and Brooks and Cowles (1905) were certain that the spermatozoa did not penetrate the eggs in the body cavity. Considering the small size of the male nucleus, however, it seems probable that *Ph. viridis* is not unusual and that the presence of two nuclei in the eggs in the body cavity may in the future be demonstrated in other species of phoronids. The fact that all phoronids investigated in this respect are alike in having the first meiotic division of ripe body cavity eggs arrested at metaphase suggests that they may also resemble one another with respect to the site of fertilization. It is not certain when the spermatozoon actually enters the egg of *Ph. viridis,* although it seems very probable that this takes place at the time of the breakdown of the germinal vesicle.

## SUMMARY

1. The seasonal changes in the gonad of *Phoronopsis viridis* are described and are seen to involve the proliferation after spawning of vasoperitoneal tissue which is subsequently reduced and largely replaced by developing reproductive tissue.

2. No significant difference between the body lengths of male and female animals was found in the group of 169 animals measured. There is thus no evidence for protandry on the basis of body length.

3. Spermatozoa were found in the nephridia of both male and female animals and in the body cavity of female animals from January to May. Ripe eggs in the body cavity, in which the female nucleus is arrested at first metaphase, also contain a male nucleus. Eggs still covered by the investing epithelium and in which the germinal vesicle is still intact do not contain a male nucleus.

4. *Ph. viridis* is considered to be probably dioecious. Spermatozoa are believed to enter the female through the nephridium and fertilization appears to take place in the body cavity of the female, following the breakdown of the germinal vesicle. The egg when spawned is already fertilized and the maturation divisions proceed as soon as it is shed into sea water.

## LITERATURE CITED

Brooks, W. K., and R. P. Cowles, 1905. *Phoronis architecta.* Its life histoy, anatomy and breeding habits. *Mem. Nat. Acad. Sci.,* **10**: 69–111.

Cori, C. J., 1890. Untersuchungen über die Anatomie und Histologie der Gattung *Phoronis. Zeitschr. Wiss. Zool.,* **51**: 480–568.

Cori, C. J., 1939. Phoronidea. Bronns' Klassen und Ordnungen des Tierreiches, 4:4:1:1. (Band 4; Abt. 4; Buch 1; Teil 1.)

De Selys Longchamps, M., 1902. Récherches sur le développement de *Phoronis. Arch. Biol., Paris,* **18**: 495–597.

De Selys Longchamps, M., 1907. *Phoronis.* Fauna u. Flora Neapel. no. 30.

Gilchrist, J. F. D., 1907. New forms of the Hemichordata from S. Africa. *Trans. S. Africa Phil. Soc.,* **17**: 151–176.

Hilton, W. A., 1930. Phoronidea from the coast of southern California. *J. Ent. Zool.,* **22**: 33–35.

Ikeda, I., 1903. On the development of the sexual organs and of their products in *Phoronis. Annot. Zool. Jap.,* **41**: 141–153.

Kowalevsky, A., 1867. Über die Anatomie und Entwicklung von *Phoronis.* Original in Russian, from translation by R. Leukhart in Ber. wiss. Leist. nied. Tiere. 1867. pp. 163–304.

Marcus, E., 1949. *Phoronis ovalis* from Brazil. *Bot. Fac. Fil., Cieno. Letr. Univ. São Paulo,* **99**; Zoologica no. 14: 157–172.

Pixel, H. L. M., 1912. Two new species of the Phoronidea from Vancouver Island. *Quart. J. Micr. Sci.,* **58**: 257–284.

Simpson, G. G., and A. Rose, 1939. Quantitative zoology. McGraw Hill Book Co. New York.

Torrey, H. B., 1901. On *Phoronis pacifica* sp. nov. *Biol. Bull.,* **2**: 283–288.

# THE X–IRRADIATION OF MARINE GAMETES.[1] A STUDY OF THE EFFECTS OF X–IRRADIATION AT DIFFERENT LEVELS ON THE GERM CELLS OF THE CLAM, SPISULA (FORMERLY MACTRA)

ROBERTS RUGH

*Radiological Research Laboratory, Columbia University, New York N. Y., and Marine Biological Laboratory, Woods Hole, Mass.*

This study was made for the following reasons:

1. To determine the radiosensitivity of the sperm and eggs of the clam and to compare it with the radiosensitivity of Arbacia gametes.
2. To determine whether cysteine or the new Bacq solution (B-mercapto-ethylamine) has any protective effect on the gametes and early development of marine forms.
3. To determine whether activation and syngamy could be separated by x-irradiation, and, if so,
4. To determine whether cross-fertilization with irradiated gametes and inter-generic parthenogenesis could be accomplished.

## MATERIALS AND METHOD

The eggs and sperm of the mollusc, *Spisula solidissima,* were used in these studies.

The eggs of the clam Spisula seem to be produced almost continuously, and (according to Allen) are available except for a period of about 10 days in August (Allen, 1951). The shell is cracked along the two-year line and the ovaries are cut out and dissected into small pieces on cheese-cloth suspended in a beaker of filtered sea water. The eggs pass through the cheese-cloth leaving behind the ovarian tissue. The ovarian masses are gently agitated to liberate the eggs. About one hour later, the sea water is decanted off and fresh (filtered) sea water is added in sufficient quantity to bathe thoroughly the eggs. This process is repeated after the eggs have settled so that the eggs get at least two thorough washings. After this they can be fertilized, or can be set aside at room temperature for use within 6–8 hours, or can be placed in the refrigerator at 10 degrees C. for use during the next 24–30 hours. The fertilization after 24 hours is still 100%, but drops to about 40% in 48 hours. Eggs in refrigeration are brought to room temperature and changed to fresh sea water before use.

The sperm are obtained by cutting the testes into small pieces in a minimum of sea water in No. 2 Stender dishes. These so-called "dry sperm" may be kept in covered dishes for dilution and use during 24 hours, or, if placed in refrigeration at 10 degrees C. may be kept for 48 hours longer. Just before use in fertilization, one drop of dry sperm is added to 40 cc. of sea water (No. 2 Stender-full) and the suspension made homogeneous with pipette stirring. Then one pipette-full of this suspension is added to a finger bowl of eggs to secure normal fertilization of all eggs. If the sperm are refrigerated, they are given about ten minutes in suspension to become thoroughly activated. In all experiments involving irradiated sperm similar dilutions were made up after irradiation and similar amounts of diluted spermatozoa were used.

---

[1] This document is based on work performed under Contract AT–30–1–GEN–70 for the Atomic Energy Commission.

The two presumably protective chemicals used were cysteine hydrochloride and another − SH solution obtained from Dr. Bacq of Bruges, known as 1573L. It is B-mercaptoethylamine. Dr. Bacq has demonstrated (1951) the protective value of this drug for mice, and claims that it is even more effective than is cysteine. The cysteine experiments have been carried on largely at the Argonne Laboratories by Patt and his co-workers (1949, 1950).

The solution obtained from Dr. Bacq is in ampoules of 10 cc. each representing 100 mg., and has been demonstrated by him to be non-toxic but protective to mice against x-irradiation when used at levels of three mg. per mouse of average weight of 30 grams (Bacq *et al.,* 1951). The undiluted solution has a pH of approximately 7.5. It was diluted in filtered sea water and adjusted to a pH of 8.0. The concentrations used were designated in milligrams per cent. It was found that 0.5 mg. % was lethal to Arbacia plutei (used as test material) and that 0.01 mg. % was non-toxic to gametes and early embryos. Twice this concentration, 0.02 mg. %, was toxic for Arbacia plutei over a 24-hour period.

Cysteine hydrochloride was obtained in the crystalline form and it was found that a 1% solution in sea water had a pH of 1.9. This solution was made up fresh for each experiment. It was adjusted with concentrated NaOH to a pH of approximately 8 which is in the range of normal sea water. X-irradiation to 18,900 r did not alter the pH of the cysteine solution. Cysteine in solution is more stable as an acid and unless kept free from air (oxygen) and at refrigeration temperatures, it develops a flocculent sediment due to degeneration to cystine. It is known that cysteine activates enzymes, combines with $H_2O_2$ (which is known to be formed in irradiation solutions) and rapidly oxidizes to the non-protective SS-cystine. It was found that a 0.5% solution caused a slight shrinking of clam eggs and a wrinkling of the surface, suggestive of hypertonicity. A 0.1% solution had no such effect and cleavage to 100% could be obtained. However, development was abnormal, so that a solution of 0.01% was used for these experiments.

In all fertilization experiments controls were set up at the beginning and at the end of each series so that the time factor of the experiment was not involved. Regulation finger bowls were used, each of which had been pre-tested for possible contamination. This was done by using sea water and living Arbacia embryos kept over a period of three days. Each bowl contained 100 cc. of filtered sea water, and all bowls were kept under the same conditions of light and temperature.

The x-ray facilities were those of the Marine Biological Laboratory, Woods Hole, Mass. The factors were 182 kv, 25 ma with two alternate parallel tubes having equivalent filtration of 0.2 mm. Cu and output of 6300 r/min., under conditions used in these experiments. When eggs or sperm were to be irradiated for more than four minutes, the plastic fly box in which they were placed was surrounded by ice cubes so that heat emanating from the filaments of the x-ray tubes would not affect the material. The plastic containers had been previously tested against living embryos for toxicity.

## Observation and Experimental Data

### *Spisula*

*Spermatozoa*

The spermatozoa of this genus are more radioresistant than are the eggs. This was determined by studying the effect of x-irradiated sperm on cleavage and on early development when used with normal, unirradiated eggs. The results were compared with those obtained when normal sperm were used with x-irradiated eggs.

Fertilization of normal eggs was accomplished to approximately 100% with concentrated (dry) spermatozoa exposed to all levels of x-irradiation from 3,150 r to 264,600 r. One can say, therefore, that the mechanics of fertilization seem to be in no way altered by the x-irradiation of spermatozoa of this species at any level studied.

Fertilizing power in all vertebrates naturally presumes motility on the part of the spermatozoa. However, motility alone is no guarantee of successful fertilization. Motility was not stopped in concentrated sperm irradiated to the maximum level of 264,600 r and then diluted. If the highly exposed spermatozoa are kept concentrated and left at room temperature (23–25 degrees C.) for 24 hours, only about 10% of the eggs are fertilized, and very few of these develop into motile trochophores. An exposure of concentrated sperm to 189,000 r followed by 24 hours in the refrigerator at 10 degrees C., did not alter the motility or the fertilizing power of Spisula spermatozoa. Many of the trochophores produced from such sperm and normal eggs appeared to be quite normal. With further refrigeration to 48 hours there was complete loss of fertilizing power by the spermatozoa. Controls, under similar conditions, gave normal fertilization and normal trochophores.

Cleavage delay of normal eggs fertilized by irradiated sperm was slightly increased with increasing exposures to x-irradiation.

Table I represents the average of six sets of readings, each of which involved thousands of eggs. Experiments were performed at the laboratory temperature of 23–25 degrees C.

Table I points up two possibilities. (1) The interval between the time of fertilization and the time of first cleavage of 50% of the eggs is increased by x-irradiation of the spermatozoa alone, but the increase is not linear nor does it follow any ratio with respect to the increased irradiation. The delay is increased to a maximum at about 113,400 r and then the curve shows a plateau of effect or even a slight decrease with further irradiation. This suggests that while the activating factors of the spermatozoon are not altered (*i.e.,* fertilization is achieved in all), the genetic material of the sperm, the syngamic or even the mitotic equipment, is so damaged that the initial cleavage is altered. The maximum delay of 15 minutes represents approximately the normal interval between the first and the second cleavages, so that possibly the first cleavage is actually omitted at 163,800 r or above. To substantiate this thesis further it might be pointed out that after the plateau is reached, there is never any reduction of the cleavage delay to a level which might be compared with the controls. Haploid (parthenogenetic) embryos have been produced among other genera, and by other means, which showed such cleavage delay and abnormalities (Fankhauser, 1945; Parmenter, 1933, 1940; Harvey, 1936, 1940; Tyler, 1941; Tyler and Bauer, 1937; Rostand, 1938; Porter, 1939; Kawamura, 1939; Tchou-Su and Chen-Chou-Hsi, 1940). It should be emphasized further that cleavage of 50% of the eggs includes all eggs fertilized, and some of these may have been fertilized by spermatozoa of varying radiation damage so that the time lapse represents an average of the functional activity of all damaged sperm. It is likely that in these eggs there is an internal maladjustment of normal chromosomal material from the egg with variously damaged chromosomal material from the x-irradiated sperm (assuming that no two sperm were necessarily damaged in the same way). In some eggs the partially damaged sperm genetic material may have little adverse effect while in others it may be completely incompatible with the normal egg complement and thereby either kill the zygote or cause developmental abnormalities (O. Hertwig, 1910, 1911; P. Hertwig, 1911, 1916; Rugh, 1939a, 1939b). Further (2) if one studies the trochophores (early embryos) arising from these fertilized eggs one finds that the low 6,300 r level of exposure as well as the very high 264,600 r exposure each produces nearly as many motile, ciliated trochophores as did the control sperm. The low point of trochophore production and maximum incidence of abnormalities is at about 163,800 r, suggesting that this level of exposure of spermatozoa causes the maximum of developmental incompatibilities. The abnormal trochophores also exhibit a wide range of sizes. Fertilization incompatibilities are overcome by further irradiation of the spermatozoa (Rugh and Exner, 1940). It is true that the trochophores from highly irradiated sperm are not normal and that they do not develop beyond 30 hours, but they are structurally quite uniform and appear to be very much like those of the controls. This may be due to the probability that these are haploid embryos. This further substantiates the thesis

TABLE I

| Sperm condition | Time from fertilization to first cleavage for 50% of eggs | Range of times | Delay in minutes | Trochophore % |
|---|---|---|---|---|
| Sea water control | 58″ | 56– 61 | 0 | 100 |
| 6,300 r | 1′02″ | 59–1′04″ | 4 | 100 |
| 12,600 r | 1′03″ | 01–1′05″ | 4 | 100 |
| 18,900 r | 1′05″ | 1′03–1′06″ | 7 | 72 |
| 25,200 r | 1′07″ | 1′05–1′08″ | 9 | 45 |
| 31,500 r | 1 09″ | 1′05–1′14″ | 11 | 62 |
| 44,100 r | 1′10″ | 1′08–1′13″ | 12 | 55 |
| 63,000 r | 1′11″ | 1′07–1′16″ | 13 | 48 |
| 89,200 r | 1′13″ | 1′07–1′17″ | 15 | 60 |
| 113,400 r | 1′12″ | 1′06–1′17″ | 14 | 45 |
| 138,600 r | 1′13″ | 1′07–1′20″ | 15 | 40 |
| 163,800 r | 1′10″ | 1′04–1′15″ | 12 | 33 |
| 170,400 r | 1′09″ | 1′05–1′14″ | 11 | 40 |
| 189,000 r | 1′10″ | 1′08–1′11″ | 12 | 65 |
| 214,200 r | 1′12″ | 1′06–1′16″ | 14 | 85 |
| 239,400 r | 1′11″ | 1′06–1′15″ | 13 | 95 |
| 264,600 r | 1′10″ | 1′06–1′15″ | 12 | 95 |

that the greater the irradiation of the sperm the less deleterious effect will the sperm genetic material have on the developmental process of the resulting zygote and embryo.

Since concentrated spermatozoa can be exposed to 264,600 r and, if properly diluted, can still fertilize 100% of the available eggs and since these in turn become ciliated and motile trochophores within 6 hours at laboratory temperatures, it seems to be difficult if not impossible to obliterate or alter the fertilizing mechanism of the spermatozoa by x-irradiation. It is presumably only the genetic (chromosomal) material that is damaged and this occurs at the lower levels of irradiation. This is not evident, however, until the developmental processes are brought into play.

All the above observations were based on the x-irradiation of dry (concentrated) spermatozoa. It was obviously in order similarly to irradiate sperm at various dilutions to compare the radiosensitivity. Here the situation is quite different.

Table II

*Fertilization percentage of normal eggs and irradiated sperm**

| Exposure | Dilutions of Sperm** | | |
|---|---|---|---|
| | 1/20 | 1/200 | 1/2000 |
| Controls | 100 | 100 | 100 |
| 6,300 r | 95 | 95 | 90 |
| 9,450 r | 95 | 95 | 80 |
| 12,600 r | 95 | 80 | 50 |
| 15,750 r | 95 | 75 | 30 |
| 18,900 r | 95 | 60 | 30 |
| 22,050 r | 90 | 45 | 20 |
| 25,200 r | 95 | 33 | 0 |
| 28,350 r | 90 | 5 | 0 |
| 31,500 r | 95 | 1 | 0 |
| Controls*** | 90 | 95 | 80 |

* Values are per cent cleavage in all eggs studied.

** The number of sperm for each batch of eggs could not be controlled accurately. Nevertheless, the same method of sampling was used throughout so that it can be assumed that the numbers of available sperm were rather constant.

*** Since the process of irradiation involved time, a second set of non-irradiated controls was used at the termination of the irradiation period. In this way the time factor above could be included.

Concentrated (dry) sperm may be used for many hours, particularly if they are kept in the refrigerator in a covered dish to prevent desiccation. The sperm dilution experiments, to the contrary, had to be conducted within a relatively short time since it is well known that mere dilution will activate spermatozoa which have a limited reserve of energy and consequently a limited life (Lillie, 1919). One cubic centimeter of fluid may be roughly considered as 20 drops. Therefore, one drop of dry sperm added to 10 cc. of sea water was considered to be a 1/200 dilution. Likewise, one drop of dry sperm added to 100 cc. of sea water would be a 1/2000 dilution. The minimum dilution of sperm used was 1/20 or one drop of dry sperm to one cc. of sea water. The same volume of sperm suspension was used in all fertilization experiments. In all cases the sea water was filtered and used at the laboratory temperature immediately. After a preliminary run it was found that irradiation of spermatozoa to dilutions of 1/2000 or more could not tolerate exposures above 31,500 r. Therefore it was decided to use graded doses of exposure up to 31,500 r only.

The results of these experiments are shown in Table II.

The more dilute the spermatozoa the more radiosensitive, as measured by the fertilizing power of irradiated sperm and normal eggs. Further, the concentrated sperm at any level produced (qualitatively) more normal trochophores than did the diluted sperm, suggesting that even the genetic material may have been more adversely affected by irradiation in the diluted state.

Presuming that the genetic contributions of highly irradiated sperm might be of no significance (based on parthenogenetic stimulation of Spisula eggs by Spisula sperm irradiated to 264,600 r), it was conjectured that such sperm might be able to stimulate the eggs of the echinoderm, Arbacia, and cause parthenogenetic development. Interspecific crosses of this type have been accomplished among the amphibia (Rugh and Exner, 1940). This, however, could not be achieved in even a single Arbacia egg in many attempts with thousands of eggs, and concentrated and irradiated Spisula sperm. The physical structure of the Spisula sperm must be such as to be incompatible with the Arbacia egg cortex.

It was noted, however, that Spisula spermatozoa irradiated to 63,000 r or more and kept for some 24 hours, even in the refrigerator, gave evidence of secretion of a mucilaginous substance which caused them to be clumped together into long strings (see Fig. 3). Such secretion and clumping did not inactivate the spermatozoa as they were seen still to be vibratile as if stuck by their tail tips.

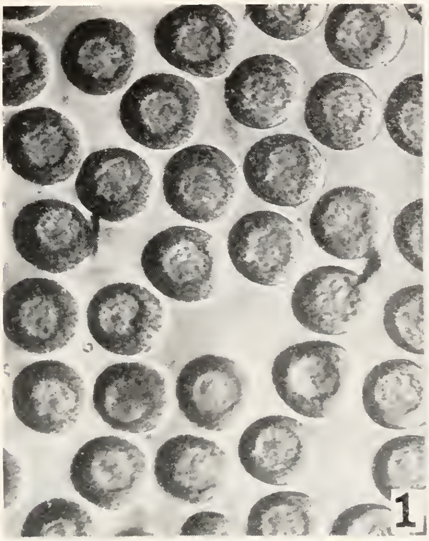
### *Spisula eggs*

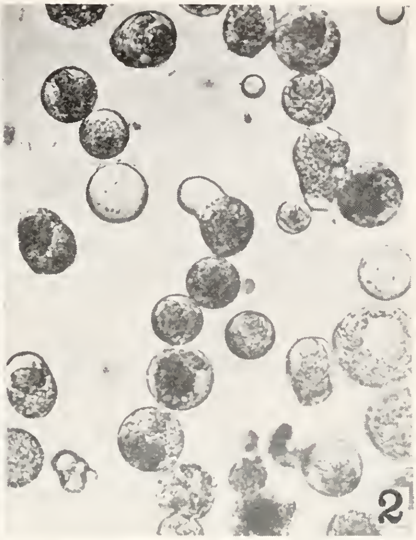
A single specimen of this clam will produce literally millions of eggs (Allen, 1951) all of which are in the germinal vesicle stage and each of which measures about 50 microns in diameter (see Fig. 1). If the eggs are properly washed at least twice, they can be used for as long as 8–10 hours at laboratory temperatures or up to 24 hours if kept in the refrigerator at 10 degrees C. Even after 48 hours at refrigeration about 40% of these eggs can be fertilized and will develop into normal larvae (trochophores).

In general the eggs of Spisula are more radiosensitive than are the spermatozoa, when measured by the ability to cleave and to develop into trochophores. An exposure of the eggs to 6,300 r is roughly equivalent to 18,900 r to the spermatozoa. Since motility cannot be a criterion of activity, the only clue of the intact egg to damage is its failure to be fertilized or to develop normally.

One might expect that the egg, with its abundant cytoplasm, would show damage more readily than would the spermatozoon which is almost devoid of cytoplasm. However a few eggs exposed to 252,000 r did cleave following fertilization with normal spermatozoa but none developed into any semblance of a larval trochophore. There is evidence of membrane and cytoplasmic damage in these eggs because most of them became ruptured and developed vesicular protrusions following fertilization with normal sperm. (Compare Figs. 1 and 2.)

X-irradiated eggs showed cleavage delay when fertilized by normal spermatozoa but the delay was more linear than when x-irradiated sperm were used to fertilize normal eggs. Also, the eggs did not tolerate the magnitude of doses used for the sperm. These data for the eggs include all eggs exposed but in the case of the spermatozoa it is impossible to determine what percentage of the invisible gametes are functional. At 3,150 r all eggs could be fertilized by normal sperm and would give rise to trochophores but at 31,500 r only some 70% of the eggs reached that





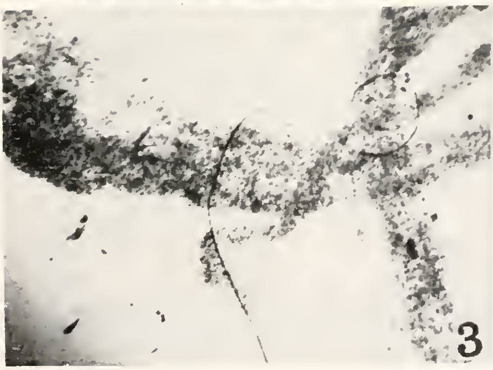


FIGURE 1. Normal eggs of Spisula, washed and ready for fertilization.

FIGURE 2. Spisula eggs exposed to 214,200 r x-rays and then fertilized by normal Spisula sperm, showing rupture and exovate masses. Some of the extruded masses and vesicles have rounded out to appear as miniature and deformed egg masses. Very few of these eggs ever cleaved, and none reached the trochophore stage.

FIGURE 3. Spisula sperm x-irradiated in cysteine which accentuated the secretion of a mucilaginous substance, causing the sperm to be aggregated into long strings.

FIGURE 4. Stringy aggregations of Spisula eggs x-irradiated in cysteine in sea water; reaction similar to that for spermatozoa (Fig. 3).

stage, even though there was 100% cleavage. The time of cleavage of 50% of x-irradiated eggs (fertilized by normal sperm) was as shown in Table III.

Spisula eggs exposed to higher levels of x-irradiation were able to cleave and develop. For instance, 90% of the eggs exposed to 94,500 r were fertilized and 40% became ciliated and motile trochophores. At 157,500 r there were also 90% blastulae and 10% trochophores and at 189,000 r there were very few cleavages and no trochophores developed.

Dilution of Spisula eggs had no appreciable effect on their radiosensitivity. The eggs were not irradiated while in the ovary because it was necessary to wash them twice in order to have them fertilizable at all. This meant that a concentration comparable to "dry sperm" (meaning gametes without any extra fluid) could not be obtained for the eggs. Nevertheless, eggs were concentrated after washing so that there was approximately a 1 : 1 ratio of eggs to sea water, and other eggs were suspended in sea water to give a 1 : 10 ratio. There was no appreciable difference in eggs irradiated in the two dilutions as measured by fertilizability and development. It is possible that even a 1 : 1 dilution (*i.e.*, the presence of any fluid) might be deleterious to eggs during x-irradiation.

TABLE III

| | Time for 50% cleavage | Delay in minutes | Trochophores at 24 hours |
|---|---|---|---|
| Control | 58″ | 0 | 100% |
| 3,150 r | 61″ | 3 | |
| 6,300 r | 62″ | 4 | 60% |
| 9,450 r | 64″ | 6 | |
| 12,600 r | 65″ | 7 | 15% |
| 15,750 r | 66″ | 8 | |
| 18,900 r | 68″ | 10 | 5–10% |
| 22,050 r | 70″ | 12 | |
| 25,200 r | 71″ | 13 | 10% |
| 28,350 r | 73″ | 15 | |
| 31,500 r | 74″ | 16 | 5% |

If eggs were irradiated and were then kept at 10° C. in the refrigerator for 24 hours and were brought to room temperature and fertilized, there was a higher percentage of cleavage and development to the trochophore stage than in eggs fertilized immediately after irradiation. It is not clear whether it was the delay or the refrigeration (or, possibly both factors) that seemed to be beneficial to the eggs. At 31,500 r some 40% ciliated trochophores appeared by 18 hours post-fertilization and at 63,000 r plus refrigeration some 50% trochophores developed. This may indicate some recovery on the part of the eggs under refrigeration, or it may indicate that toxic substances produced during irradiation in the medium in which the eggs were suspended had been dissipated.

There was some evidence of nuclear damage by exposure to 6,300 r since only about 60% of the eggs at this level reached the trochophore stage. The nuclear damage must have been most severe at 31,500 r (with 5% trochophore development) because as many as 40% trochophores again appeared following 94,500 r. Since further irradiation above 94,000 r did not improve trochophore production, even though the change was still high, it may be presumed that cytoplasmic damage may have complicated the situation so that development could not proceed.

Spisula eggs exposed to various doses of x-irradiation were then submitted to normal spermatozoa of the echinoderm, Arbacia, pre-tested against eggs of Arbacia. At no level was a single irradiated Spisula egg fertilized by an Arbacia spermatozoon. At 264,600 r the Spisula eggs are ruptured by fertilization with normal Spisula sperm and develop ex-ovate masses, and disintegrate. If, however, such eggs are exposed to Arbacia sperm, these abnormal conditions do not occur, indicating that the Arabacia sperm are unable to penetrate the membrane and cortex even of these moribund eggs of Spisula.

In contrast with the irradiation of Spisula sperm, therefore, the egg is less tolerant and at the maximum exposure used (264,600 r) the egg membrane and cytoplasm were so damaged that fertilization was virtually impossible. The sperm, exposed to this dose, fertilized normal eggs to nearly 100%. With respect to the trochophores developing from irradiated sperm or irradiated eggs, there was no detectible difference.

### *Observations on Arbacia Gametes*

Henshaw (1940) long since completed an exhaustive study of x-irradiation of Arbacia gametes. Nevertheless, as a parallel to the above Spisula experiments a few additional observations were made on Arbacia material.

Arbacia dry sperm x-irradiated to as much as 189,000 r were added to normal Arbacia egg suspensions and brought about normal cleavage in all eggs. Henshaw (1940) claimed obliteration of fertilization after exposure of the dry sperm to 300,000 r or more. However, 6 hours after such irradiation the "dry" Arbacia sperm, when diluted, were non-motile and could not be used for fertilization. This was not true for the similarly concentrated Spisula sperm which survived many hours post-irradiation, and even longer periods under refrigeration.

At 126,000 r about 50% of the embryos from irradiated sperm became blastulae and following 189,000 r and 252,000 r only occasional blastulae developed when the dry Arbacia sperm were irradiated, and used to fertilize normal eggs.

Even though motile after exposure to 189,000 r, the Arbacia sperm were never successful in fertilizing (activating) the normal eggs of Spisula. This interphyletic cross by irradiated sperm has not been attempted previously. It was thought that such sperm, with their genetic complement presumably damaged, might act as parthenogenetic agents and stimulate Spisula eggs at least to membrane elevation and cleavage. This, apparently, does not occur so that other incompatibilities must be present.

The cleavage delay following irradiation of Arbacia eggs (first reported by Henshaw, 1932 and Henshaw and Francis, 1933) was clearly substantiated. Further, Arbacia eggs exposed to x-rays at any level up to 126,000 r could not be fertilized by normal Spisula sperm. Again this suggests incompatibility apart from nuclear considerations since the irradiated nuclear material of the Arbacia egg was never reached by the normal Spisula sperm. Control situations, in which normal Spisula sperm were used with normal Arbacia eggs, never gave cleavage either. It was presumed that irradiated Arbacia eggs might have lost some of their resistance to foreign sperm. This was not borne out.

Arbacia plutei exposed to as little as 9,450 r very quickly aggregated into sticky clumps. This stickiness occurred soon after irradiation but did not last very long. It was probably due to a secretion from the irradiated plutei, for a similar stickiness has been reported for testicular chromosomes in other aquatic forms (Rugh, 1950). Plutei tolerated as much as 44,100 r with no more deleterious effects than a retardation in growth over a period of 7 days. The pluteus is therefore much less sensitive than is either gamete.

The original incentive for these studies was to determine whether chemical agents, previously demonstrated as having some protective value for mice against x-irradiation damage, might likewise have some protective effect on marine gametes and early embryos. The substances used were cysteine hydrochloride (Patt *et al.*, 1949, 1950) and another – SH compound known as B-mercaptoethylamine (Bacq *et al.*, 1951). Both substances have been reported to give protection to mice by injection before irradiation.

*Cysteine*

It was soon found that 0.5% cysteine was hypertonic and toxic to the eggs of Spisula; that a 0.1% solution allowed fertilization and cleavage but caused developmental abnormalities and that a 0.01% solution was toxic to developmental stages over extended periods but was not toxic for short periods to the gametes and early embryos of Arbacia. Fertilization could be accomplished to a normal degree in such a concentration of cysteine. Since exposure to cysteine was of rather short duration before, during and sometimes after irradiation, the concentration of 0.01% was used throughout the experiments.

Spisula eggs immersed in a 0.01% solution of cysteine in sea water (adjusted to pH 8.0) for as much as three hours before and during x-irradiation, had no effect whatever on fertilizability of the eggs, nor on the cleavage and early development. That is, both cleavage percentage and time were normal, similar to the controls which did not have the exposure to cysteine but did have the irradiation. Those eggs which were left in the solution in which they were irradiated, or were transferred to fresh cysteine, did not benefit by the presence of the cysteine with respect to developmental rate or degree when compared with the non-cysteine controls. In fact, if left more than a few hours no Spisula embryos reached the trochophore stage even though some of the non-irradiated controls did. Only when the cysteine concentration was reduced to 0.001% was there larval survival, but never to a degree of better than the non-cysteine controls. Exposure to cysteine before, during and after irradiation for a total of 1¼ hours did not affect later trochophore development. Eggs or embryos placed in previously irradiated cysteine were not thereby protected.

There was one bit of evidence of some cysteine effect. As stated above, when gametes or the larvae are x-irradiated to certain levels, they exude a sticky substance which causes them to aggregate (Figs. 3 and 4). This tendency is accentuated by the presence of cysteine. However, cysteine alone does not cause this and while x-irradiation does, the stickness is much more extensive when living material is irradiated in the cysteine medium. Further, a lower exposure (6,300 r) of the eggs or plutei to x-rays causes the stickiness in cysteine while it does not do so without the cysteine. The aggregations under x-rays alone are in small clumps at first. They appear as long and heavy strings in x-ray plus cysteine conditions.

While cysteine does not have any effect on cleavage time or percentage, and it does not in any way seem to protect the gametes or early embryo against x-radiation, its presence does seem to cause a rounding out of the blastomeres of the two- and four-cell stages of both Spisula and Arbacia, stretching the intercellular connections so as to make them appear as though the blastomeres are almost unrelated. Many of the two-cell stages are almost separated. This is a situation one finds experimentally when such eggs are kept in a calcium-free or low-calcium medium.

### *B-Mercaptoethylamine (Bacq Solution)*

This synthetic solution has a pH of 7.5 which is very close to that of normal sea water. It was adjusted to pH 8 before use. Bacq used 3 mg. per 30 gm. mouse, injected intraperitoneally, to give protection. This changed mortality of mice exposed to 700 r x-rays from 93% to 5%. When diluted to 0.1 mg. % in filtered sea water it was found to be lethal to Arbacia plutei; at 0.04 mg. % it was not toxic to the plutei but was toxic to the unfertilized eggs, causing a cortical wrinkling. The concentration which was not toxic, and which was finally used, was 0.01 mg. % at pH 8.0.

There was some slight statistical evidence that this solution allowed more eggs to develop and to develop further, following irradiation in the solution, than did the control situation of irradiation in sea water alone. There was also some slight evidence of acceleration of early cleavage. These were gross impressions which would have to be checked under highly controlled conditions of temperature and concentrations. Even then they would not be very significant, if confirmed, because the protection, if any, was so slight.

For instance, Arbacia eggs irradiated in the Bacq solutions did show a slightly higher percentage of development and the larvae developed farther than in the irradiated sea water controls. Also, Spisula eggs exposed to 18,900 r in the Bacq solutions survived to the trochophore stage better and in larger number than did their controls. But the degree of improvement in either case did not encourage an exhaustive statistical study since it was positive but not significant. One might say that if either solution showed any protection during irradiation it was the Bacq solution. In neither solution was development allowed to proceed normally very far.

## SUMMARY AND CONCLUSIONS

1. The spermatozoa of the clam Spisula are more radioresistant than are its eggs.

2. The fertilizing power of Spisula sperm in the dry or concentrated state could not be affected by x-irradiation even to 264,000 r.

3. Increasing x-irradiation of Spisula spermatozoa caused increasing delay in cleavage time of normal eggs fertilized by such sperm, but the curve was not linear and did not exceed 15 minutes. This delay represents the time interval between the first and the second normal cleavages.

4. Trochophore production was at its lowest following 163,000 r x-irradiation of the spermatozoa, but with further x-irradiation trochophore production reached 95%. Such trochophores, while viable for a time, were not normal.

5. Dilution of Spisula spermatozoa increased their radiosensitivity as determined by the effect on subsequent embryonic development.

6. The demonstrated parthenogenetic stimulating ability of Spisula spermatozoa exposed to 189,000 r or more x-rays and used with Spisula eggs, could not be achieved when Spisula sperm were used with Arbacia eggs.

7. X-irradiated Spisula gametes exude a mucilaginous substance which causes them to aggregate (clump).

8. Very few Spisula eggs exposed to 189,000 r x-rays cleaved and none became trochophores. After 214,200 r there was evidence of cytoplasmic and membrane destruction.

9. Neither cysteine hydrochloride or B-mercaptoethylamine appeared to give any appreciable protection to Spisula gametes against x-irradiation as determined by the subsequent development of the embryo.

## LITERATURE CITED

Allen, R., 1951. The use of *Spisula solidissima* eggs in cell research. *J. Cell. and Comp. Phys.*, **37**: 1–2.

Bacq, Z. M., A. Herve, J. Lecomte, P. Fisher and J. Blavier, 1951. Protection contre le rayonnement x par la-B-mercaptoethylamine. *Arch. int. de Physiologie*, **59**: 442–447.

Fankhauser, G., 1945. The effects of changes in chromosome number on amphibian development. *Quart. Rev. Biol.*, **20**: 20–72.

Harvey, E. B., 1936. Parthenogenetic merogony or cleavage without nuclei in *Arbacia punctulata*. *Biol. Bull.*, **71**: 101–121.

Harvey, E. B., 1940. Development of half-eggs of *Arbacia punctulata* obtained by centrifuging after fertilization, with special reference to parthenogenetic merogony. *Biol. Bull.*, **78**: 412–427.

Henshaw, P. S., 1932. Studies of the effect of roentgen rays on the time of the first cleavage in some marine invertebrate eggs. I. Recovery from roentgen ray effects in Arbacia eggs. *Amer. J. Roentgen. Rad. Therapy*, **27**: 890–898.

Henshaw, P. S., 1940. Further studies on the action of roentgen rays on the gametes of *Arbacia punctulata*. I. Delay in cell division caused by exposure of sperm to roentgen rays. II. Modification of the mitotic time schedule in the eggs by exposure of the gametes to roentgen rays. VI. Production of multipolar cleavage in eggs by exposure of the gametes to roentgen rays. *Amer. J. Roentgen. Rad. Therapy*, **43**: 899–933.

Henshaw, P. S., and D. S. Francis, 1933. The effect of x-rays on cleavage in Arbacia eggs; evidence of nuclear control of division rate. *Biol. Bull.*, **70**: 28–35.

Hertwig, O., 1910. Die Radiumstrahlung in ihrer Wirkung auf die Entwicklung tierischer Eier. *Sitz. K. Pruss. Akad. Wissensch.*, 24 Feb. and 28 Juli.

Hertwig, O., 1911. Die Radiumkrankheit tierischer Keimzellen. Ein Beitrag zur experimentellen Zeugungs- und Vererbungslehre. *Arch. mikrosk. Anat., II Abt.*, **77**: 1–164.

Hertwig, P., 1911. Durch Radiumbestrahlung hervorgerufene Veränderungen in den Kernteilungsfiguren der Eier von *Ascaris megalocephala*. *Arch. mikrosk. Anat.*, **77**: 301–311.

Hertwig, P., 1916. Durch Radiumbestrahlung verursachte Entwicklung von halbkernigen Triton- und Fischembryonen. *Arch. mikrosk. Anat., II Abt.*, **87**: 63–122.

Kawamura, T., 1939. Artificial parthenogenesis in the frog. I. Chromosome numbers and their relation to cleavage histories. *J. Fac. Sci. Hiroshima Univ.*, **6**: 115–218.

Lillie, F. R., 1919. Problems in fertilization. Univ. Chicago Press, Chicago, Illinois.

Parmenter, C. L., 1933. Haploid, diploid, triploid, and tetraploid chromosome numbers, and their origin in parthenogenetically developed larvae and frogs of *Rana pipiens* and *R. palustris*. *J. Exp. Zool.*, **66**: 409–453.

PARMENTER, C. L., 1940. Chromosome numbers in *Rana fusca* parthenogenetically developed from eggs with known polar body and cleavage histories. *J. Morph.,* **66**: 241–260.

PATT, H. M., E. B. TYREE, R. L. STRAUBE AND D. E. SMITH, 1949. Cysteine protection against x-irradiation. *Science,* **110**: 213–214. (See also ANL 4291 and 4333.)

PATT, H. M., D. E. SMITH, E. B. TYREE AND R. L. STRAUBE, 1950. Further studies on modification of sensitivity to x-rays by cysteine. *Proc. Soc. Exp. Biol. Med.,* **73**: 18–21.

PORTER, K. R., 1939. Androgenetic development of the egg of *Rana pipiens. Biol. Bull.,* **77**: 233–257.

ROSTRAND, J., 1938. La parthénogènes des vertébrés. Actualites Scientifiques et Industrielles, No. 651, pp. 3–62. Herman et Cie., Paris.

RUGH, R., 1939a. Developmental effects resulting from exposure to x-rays. I. Effect on the embryo of irradiation of frog sperm. *Proc. Amer. Philos. Soc.,* **81**: 447–471.

RUGH, R., 1939b. The effect on the embryo of x-radiation of spermatozoa. *J. Contraception,* **4**: 123–125.

RUGH, R., 1950. The immediate and delayed morphological effects of x-irradiation on meiotic chromosomes. *J. Cell. Comp. Physiol.,* **36**: 185–203.

RUGH, R., AND F. EXNER, 1940. Developmental effects resulting from x-rays. II. Development of leopard frog eggs activated by bullfrog sperm. *Proc. Amer. Philos. Soc.,* **83**: 607–619.

TCHOU-SU, M., AND CHEN-CHOU-HSI, 1940. The technique of Professor Bataillon and some three year old parthenogenetic frogs. *Chinese J. Exp. Biol.,* **1**: 303.

TYLER, A., 1941. Artificial parthenogenesis. *Biol. Rev.,* **16**: 291–336.

TYLER, A., AND H. BAUER, 1937. Polar body extrusion and cleavage in artificially activated eggs of *Urechis caupo. Biol. Bull.,* **73**: 164–180.

# PHYSIOLOGICAL ANALYSIS OF THE CORTICAL RESPONSE OF THE SEA URCHIN EGG TO STIMULATING REAGENTS.
## I. RESPONSE TO SODIUM CHOLEINATE AND WASP–VENOM

MASAO SUGIYAMA

*The Sugashima Marine Biological Station of Nagoya University, Sugashima, Shima-gun, Mie-Ken, Japan*

The importance of the cortical response of sea urchin eggs in the process of fertilization or artificial activation has long been recognized and attention has been devoted to this problem. Loeb (1913) stated that the initiation of development in the sea urchin egg is due to a change in the surface of the egg. He maintained the theory, also, that cytolysis of the cortical layer generally results in membrane formation. According to Just (1919), some cortical change, beginning at the point of sperm-entry, sweeps over the egg before the actual elevation of the fertilization membrane. Gray (1922) postulated that the essential change of the egg surface is complete before any visible change is possible. Moser (1939) has found that after fertilization, the cortical granules embedded in the cortex of the Arbacia egg start to break down in a wave-like fashion and cause the formation of the fertilization membrane. He suggests that the breakdown of the cortical granules follows a preliminary release of calcium which may travel in a rapid wave-like fashion around the egg cortex. More recently, many interesting facts as to fertilization and activation are being found by the investigators of Runnström's institute.

However, despite great advances in our knowledge on this problem, it cannot yet be said that we have any sufficient understanding of the process of fertilization and activation. The present paper is the first of a series presenting the results of experiments which have been made in the hope of analyzing further the cortical response of the sea urchin egg.

## Material and Methods

The sea urchin chosen for these experiments was *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus,* the spawning season of which extends over the whole winter. This species was used because the cortical granules of the egg are easily visible in the living state without centrifuging. With regard to the cortical granules of this species, Motomura (1941) studied them in detail, designating them "Janus green granules."

The eggs were secured in the usual manner. In all experiments only such egg material as showed good fertilizability in control experiments was used. For the first series of experiments a 1 per cent solution of sodium choleinate (Grübler) was made with sea water, sufficient amounts being added to sea water to make the desired solutions. The stock solution became ineffective in a few days but could be used for at least two days at room temperature (10–13° C.). The pH of the sodium choleinate solution was 8.1–8.3. For the second series of experiments, wasp-venom was obtained from *Polistes jadwigae*. Cutting the abdomen of the wasp, the poison gland was taken out and dried in a desiccator. At the time of an experiment, it was placed in a few drops of sea water and venom was extracted. The concentration of wasp-venom was difficult to determine. All the experiments were made at room temperature.

## EXPERIMENTS WITH SODIUM CHOLEINATE

First, some typical experiments in which sodium choleinate was used to induce the formation of the fertilization membrane will be reported. Unfertilized eggs were put into solutions of sodium choleinate of various concentrations and their response was observed. It was found that sodium choleinate was effective in inducing the formation of the fertilization membrane, and the mode of response of the eggs was somewhat different from that obtained in experiments with butyric acid or urea solution. One of the results of the experiments is shown in Table I.

TABLE I

*Formation of the fertilization membrane by means of sodium choleinate*
*(temp. 11° C.)*

| Number of drops of Na-choleinate solution added to 1 cc. of sea water | Mode of membrane formation |
|---|---|
| 20 | The membrane is violently separated in 10 seconds (Fig. 1, a). |
| 15 | The membrane is violently separated in 40 seconds (Fig. 1, a). |
| 10 | "Normal" membrane formation took place in 50% of the eggs within 2 minutes, while some eggs formed only partially (Fig. 1, b, c). After 3 minutes, 92% of the eggs had completed membrane elevation. |
| 5 | No response was observed in most of the eggs after 5 minutes. Partial membrane formation occurred in a few eggs (Fig. 1, c). |
| 4 | No response was observed in any of the eggs after 5 minutes. |

At the optimum concentration of sodium choleinate, formation of a normal-appearing fertilization membrane occurred within a few minutes. However, the mode of membrane elevation was rather different from that which takes place when the eggs are treated with butyric acid or urea solution. When such eggs are returned to sea water, roughening of the whole surface takes place within several seconds. But in the experiments with sodium choleinate, roughening of the whole surface of the eggs did not occur before membrane elevation. The membrane began to rise in one or several places, and a few minutes were required to complete its full elevation.

Too high concentration of sodium choleinate resulted in violent separation of the membrane and subsequent cytolysis. When a solution of sodium choleinate of too low concentration was used, no response was observed in most of the eggs, but partial separation of the membrane sometimes took place in a few eggs. Such par-

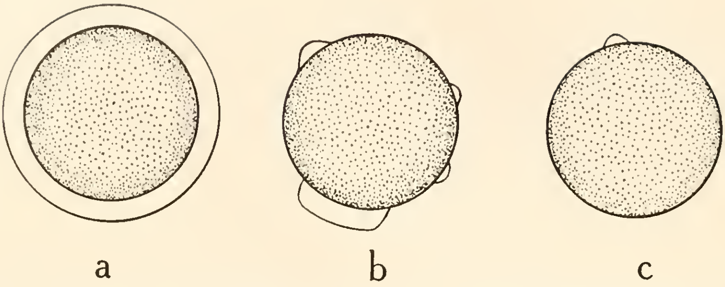


FIGURE 1. Eggs treated with sodium choleinate. a, an egg with completely elevated membrane; b and c, eggs with partially elevated membranes.

tially separated membranes remained as such even after twenty minutes' immersion in the solution.

In the next experiments, eggs treated with sodium choleinate solution of the optimum concentration (1 cc. of sea water plus 10 drops of 1% sodium choleinate solution) were washed with normal sea water when a partial separation of the membrane had just taken place. It was found that in such eggs membrane separation stopped and no further progress of the cortical change was observed. Upon examining the eggs with the oil immersion objective, it became obvious that the breakdown of the cortical granules occurred only in the part where the membrane had separated and the granules were intact elsewhere (Fig. 2). It was also found that when such eggs were inseminated after several washings with sea water, fertilization took place and the membrane separated all over the egg surface within a few minutes. The cortical granules of these eggs were thoroughly broken down. Most of these eggs, however, showed polyspermic development.

When unfertilized eggs are insufficiently treated with butyric acid, the fertilization membrane is sometimes elevated eccentrically or very slightly, but such a

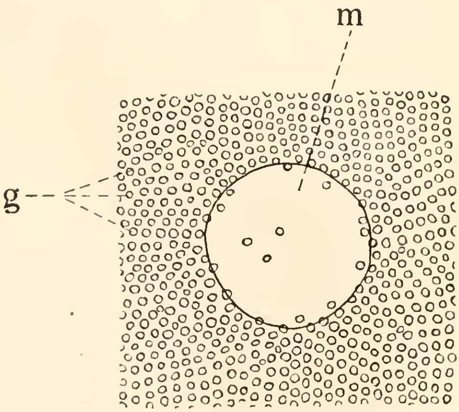


FIGURE 2. The cortex of the egg with partially elevated membrane. The cortical granules are broken down only in the area where the membrane is elevated. g, cortical granules; m, partially elevated membrane.

strictly partial separation of the membrane as in the case of sodium choleinate seldom occurs. It is interesting that the cortical response to an insufficient treatment with sodium choleinate is somewhat different from that to an insufficient treatment with butyric acid.

## Experiments with Wasp-venom

Öhman (1944) reported that a membrane similar to a normal fertilization membrane was formed under the action of bee-venom. The experiments with bee-venom have thereafter been repeated and extended by Runnström (1947), and interesting results, especially concerning the behavior of the cortical material, have been obtained.

The present author has found that there is something in common between the actions of wasp-venom and sodium choleinate. If the unfertilized eggs of *Hemicentrotus pulcherrimus* are exposed to the action of appropriate concentrations of wasp-venom in sea water, small blisters appear at several places on the egg surface and soon an elevation of the membrane begins. If the action of the wasp-venom is continued longer, a complete membrane is formed in a few minutes. However, if the eggs are washed with sea water when an elevation of the membrane has occurred only locally and not over the whole egg surface, the membrane formation is interrupted as in the experiments with sodium choleinate. If these are observed with the oil immersion objective, it is found that the cortical granules have been broken down in the part of the egg cortex where the membrane is separated, while they are intact in the remaining areas.

Runnström has reported that when the treatment with bee-venom was interrupted by washing with sea water the membrane formation in Psammechinus eggs took place almost to the same extent as under continuous action of the bee-venom. The same results were obtained in Hemicentrotus eggs also, when a high concentration of wasp-venom was used. But a partial elevation of the membrane took place when an appropriate concentration of wasp-venom was used.

## Discussion

It is well known that the first remarkable change of the sea urchin egg at the time of fertilization is, in general, a roughening process at the surface. Moser (1939) found in Arbacia eggs that this phenomenon is a consequence of the breakdown of the cortical granules. He found also that in from 10 to 20 seconds after insemination the cortical granules start to break down in a wave-like fashion, beginning at the site of sperm-entry and ending at the opposite pole of the egg. A similar behavior of the cortical granules is also observed in Hemicentrotus eggs (Motomura, 1941).

It has been suggested by several workers that some sort of invisible change is complete before any visible change occurs at the time of fertilization. This suggestion has generally been made on the basis of the fact that the block to polyspermy is rapidly established with the attachment or entry of one spermatozoon. According to Just, for instance, the "wave of negativity" progressively sweeps over the egg from the point of sperm-entry, preceding the actual beginning of membrane lifting, so that before the membrane begins to lift sperm can no longer enter at any point of the egg surface. It might be postulated that the breakdown of the cortical granules follows the so-called wave of negativity. But there has been no evidence to prove causality between them. There may be some possibility that the wave of negativity is not connected with the breakdown of the cortical granules.

If we assume that the breakdown of one cortical granule does not automatically induce the breakdown of its neighbours, then the granule breakdown must be the consequence of a more profound cortical change of a self-propagating nature. The results presented in this paper make it possible to discuss some problems on the process of the breakdown of the cortical granules. If eggs treated with sodium choleinate or wasp-venom are washed with sea water when elevation of the membrane has occurred only at several places on the egg surface, the membrane formation is interrupted. It has been noted that the cortical granules are broken down in the parts of the egg cortex from which the membrane is separated, while they are intact in the remaining areas. It is evident that the failure of the granular breakdown is not due to some impairing effect of the reagents, because, as stated above, the remaining granules are broken down if insemination is carried out afterwards. These facts constitute the evidence for concluding that the breakdown of the cortical granules itself at any point does not become the cause of the breakdown of the neighbouring granules. It seems quite possible, therefore, that some invisible cortical change of a propagating nature must occur in order that the cortical granules are broken down at the time of fertilization. Whether such an invisible cortical change is identical with Just's "wave of negativity" or not, is still obscure, because there has been no evidence to indicate that the mechanism to exclude supernumerary spermatozoa is the cause of breakdown of the cortical granules.

The work of Yamamoto (1944a, 1944b) on the eggs of the fish, *Oryzias latipes,* has demonstrated that the first visible change at fertilization is the wave-like breakdown of alveoli which are embedded in the cortical protoplasm of ripe unfertilized eggs. He postulated that a reaction of some kind, or an impulse, may be provoked by the sperm and conducted in a wave-like fashion, causing such alveolar breakdown. He termed this invisible wave the "fertilization-wave." The Oryzias egg is much larger than the sea urchin egg and its structure and nature differ from those of the sea urchin egg to a considerable degree. But the processes of the fertilization reaction in the two animals seem to have something in common with each other. The invisible cortical change in the sea urchin egg resembles the "fertilization-wave" in Oryzias eggs in its self-propagating nature, in its occurrence prior to the breakdown of the cortical structure and in its function of causing the breakdown of the cortical structure. Therefore, the author wishes to call the invisible cortical change in sea urchin eggs, also, the "wave of fertilization" or the "fertilization-wave."

As suggested elsewhere in this article, the cortical response to an insufficient treatment with sodium choleinate is somewhat different from that to an insufficient treatment with butyric acid. By means of sodium choleinate a partial separation of the membrane can be induced. This suggests that sodium choleinate causes the breakdown of the cortical granules without intervention of the fertilization-wave. This suggestion is also applicable to wasp-venom. On the other hand, such a strictly partial separation of the membrane as in the case of sodium choleinate seldom occurs as the result of an insufficient treatment with butyric acid. This probably indicates that butyric acid gives rise to the fertilization-wave, indirectly inducing the breakdown of the cortical granules. The data seem insufficient to warrant a more extended analysis, but indicate the desirability of a more refined approach to the problem of the response of the eggs to parthenogenetic reagents.

The author wishes to express his gratitude to Prof. T. Yamamoto, Dr. Jean C. Dan and Mr. E. Nakano for their valuable suggestions and advice concerning the experiments and the manuscript.

## SUMMARY

1. The formation of the fertilization membrane in Hemicentrotus eggs can be induced by an appropriate treatment with sodium choleinate or wasp-venom. When the concentration is optimum, the membrane begins to rise in one or several regions of the egg surface and several minutes are required to complete its full elevation.

2. If the eggs are washed with sea water when elevation of the membrane has occurred only locally and not over the whole egg surface, the membrane formation is interrupted. The cortical granules have been broken down only in the part of the egg cortex where the membrane is separated while they are intact in the remaining areas.

3. These results constitute the evidence for the conclusion that the breakdown of the cortical granules itself at any point does not become the cause of the breakdown of the neighbouring granules. It seems to follow, therefore, that some invisible cortical change of a propagating nature must occur in order that the cortical granules be broken down at the time of fertilization. This invisible cortical change may be called the "wave of fertilization" or the "fertilization-wave," since it resembles Yamamoto's "fertilization-wave" in Oryzias eggs.

4. It is suggested that sodium choleinate and wasp-venom cause the breakdown of the cortical granules without intervention of the fertilization-wave.

## LITERATURE CITED

Gray, J., 1922. A critical study of the facts of artificial fertilization and normal fertilization. *Quart. J. Micro. Sci.*, **66**: 419–437.

Just, E. E., 1919. The fertilization reaction in *Echinarachnius parma*. I. Cortical response of the egg to insemination. *Biol. Bull.*, **36**: 1–10.

Loeb, J., 1913. Artificial parthenogenesis and fertilization. Chicago.

Moser, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. I. Response to insemination. *J. Exp. Zool.*, **80**: 423–445.

Motomura, I., 1941. Materials of the fertilization membrane in the eggs of echinoderms. *Sci. Rep. Tohoku Imp. Univ., B,* **16**: 345–363.

Öhman, L. O., 1944. On the lipids of the sea-urchin egg. *Ark. för Zool.,* **36A, No. 7**: 1–95.

Runnström, J., 1947. Further studies on the formation of the fertilization membrane in the sea-urchin egg. *Ark. för Zool.,* **40A, No. 1**: 1–19.

Yamamoto, T., 1944a. Physiological studies on fertilization and activation of fish eggs. I. Response of the cortical layer of the egg of *Oryzias latipes* to insemination and to artificial stimulation. *Annot. Zool. Jap.,* **22**: 109–125.

Yamamoto, T., 1944b. Physiological studies on fertilization and activation of fish eggs. II. The conduction of the "fertilization-wave" in the egg of *Oryzias latipes*. *Annot. Zool. Jap.,* **22**: 126–136.

# PHYSIOLOGICAL ANALYSIS OF THE CORTICAL RESPONSE OF THE SEA URCHIN EGG TO STIMULATING REAGENTS.
## II. THE PROPAGATING OR NON-PROPAGATING NATURE OF THE CORTICAL CHANGES INDUCED BY VARIOUS REAGENTS

MASAO SUGIYAMA

*The Sugashima Marine Biological Station of Nagoya University, Sugashima*
*Shima-gun, Mie-Ken, Japan*

In the previous paper of this series, it was concluded that an invisible change of a propagating nature must occur at the time of fertilization in sea urchin eggs in order that the cortical granules be broken down. This change was called the "fertilization-wave," as in the case of Oryzias eggs (Yamamoto, 1944). It was suggested that sodium choleinate and wasp-venom do not initiate the fertilization-wave, although they exert their influence upon the cortical granules to induce their breakdown. This means that the artificially induced breakdown of the granules does not always reveal itself as a consequence of the fertilization-wave. The question then arises as to the presence or absence of the fertilization-wave in the cortical changes induced by various other artificial stimuli. The possibility must be considered that some stimulating reagents provoke the fertilization-wave, which leads to the breakdown of the cortical granules, while others act directly to cause the breakdown of the cortical granules, without inducing a fertilization-wave.

An investigation has been made to determine whether the effects of various stimulating reagents are of a propagating nature. If they are of a propagating nature, it may very probably indicate that the fertilization-wave is provoked. On the contrary, if they are of a non-propagating nature, it may be clear that the fertilization-wave does not occur. It is the purpose of the present paper to report the results of these experiments.[1]

## MATERIAL AND METHODS

The sea urchin, *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* was used throughout. The eggs to be used for experiments were carefully collected in the usual manner and only such eggs as showed good fertilizability in control experiments were used. Wasp-venom, sodium choleinate, urea, glycerine, sucrose, distilled water, detergents and fatty acids were employed as stimulating reagents.

In order to investigate whether the effect of a stimulating reagent is of a propagating nature, the following method was adopted. The egg surface was partially exposed, as described below, to the stimulating reagent for an appropriate time and then washed with sea water. The unexposed surface was examined with the oil-immersion objective. When the cortical granules in the exposed part were broken down and those in the unexposed part were intact, it was considered that the effect of the reagent was not of a propagating nature. But when the cortical granules in the whole surface, including the unexposed part, were broken down, the effect of the reagent was conceived to be of a propagating nature.

---

[1] It may be added that elaborate works on conduction of the block to polyspermy have been done by Rothschild and Swann (*Exp. Cell Research,* **2**: 137, 1951; *J. Exp. Biol.,* **28**: 403–416, 1951; *J. Exp. Biol.,* **29**: 469–483, 1952).

Partial exposure of the egg surface to the reagent was achieved by means of the technique diagrammed in Figure 1a. The jelly-coats of the eggs were first removed by treating the eggs with a weak solution of $HCl$ in sea water. It was ascertained that the eggs thus deprived of their jelly-coats were fertilizable. A small drop of sea water containing the jelly-less eggs was placed on a slide. A very small amount of vaseline was put on the four corners of a cover-glass and this was placed over the egg-sea water drop. Pushing the cover-glass with a fine needle, the eggs were pressed down and flattened until their diameter reached 160 $\mu$. Since the normal spherical egg has a diameter of about 98 $\mu$, the flattened eggs were considered to take the form schematized in Figure 1b. The upper and lower parts of the eggs adhered closely to the glass-surface, while the equatorial region, so to speak, was in contact with the sea water. It was found possible to control

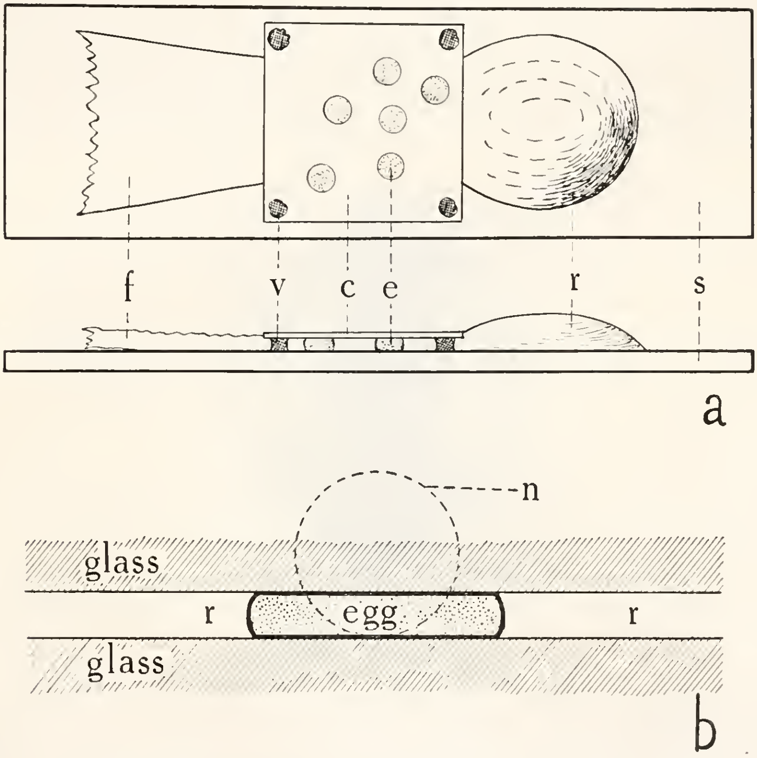


FIGURE 1. a, Diagrammatic view of the arrangement used for the experiments. b, Diagrammatic view of the compressed egg. c, cover-glass; e, egg; f, filter-paper; n, outline of a normal egg; r, reagent to stimulate eggs; s, slide-glass; v, vaseline.

the amount of vaseline so that there was no movement of the eggs during the subsequent procedure.

Several drops of the reagent to be tested were placed on the slide at one side of the cover-glass (Fig. 1a). A small piece of filter paper was applied to the other side of the cover-glass so that the reagent was drawn into the space beneath the cover-glass. After an appropriate time, the reagent was replaced by normal sea water in a similar way. Thus the equatorial region of the eggs was exposed to the reagent, while the upper and lower regions were not influenced directly by the reagent, since they were closely applied to the glass-surfaces. The cortical granules in the upper part of the eggs were examined with the oil-immersion objective. The appropriate time of exposure of the eggs to each reagent was preliminarily determined by experiments using eggs in watch glasses. The length of time sufficient to induce the breakdown of the cortical granules in the watch glasses was taken as the appropriate exposure time for the experiments.

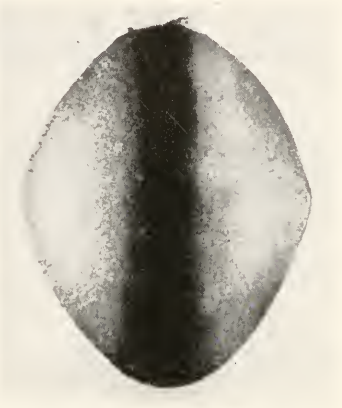


FIGURE 2. An egg, the equator of which was exposed to a solution of Janus green by the method shown in Figure 1.

Experiments were usually repeated more than twenty times for each reagent and the results were found to be quite reproducible. Figure 2 shows an egg, the surface of which was partially exposed to a solution of Janus green by the above-described method. The equatorial region is deeply stained while the other areas remain unstained, indicating that the dye did not soak into these regions. The fact that, as will be shown later, even surface-active substances such as detergents do not exert their influence on the upper part of the eggs indicates that this technique is suitable for the present purpose.

## RESULTS

### *Experiments with wasp-venon, sodium choleinate and detergents*

Wasp-venom was obtained from *Polistes fadwigae.* The poison gland was placed in a few drops of sea water and the venom was extracted. Employing the above-described technique, the equatorial regions of the eggs were exposed to the solution of wasp-venom in sea water. With an appropriate concentration of wasp-venom, the cortical granules in the equatorial region soon began to break down. After they had completely broken down, the upper part of the eggs was examined. It was found that the cortical granules in that part were quite intact and no sign of the influence of the wasp-venom was observed (Fig. 3b). If the solution was replaced by normal sea water after the membrane was elevated on the equatorial region and before cytolysis took place, no cortical change was observed in the upper part of the eggs even after 10 minutes.

Similar experiments were performed using sodium choleinate. To 1 cc. of sea water were added 10 drops of a 1 per cent solution of sodium choleinate. The result with this solution was found to be quite the same as that with wasp-venom.

In the next experiments, the effects of Monogen and Lipon were studied. Monogen is a detergent, consisting chiefly of a mixture of myristyl sulphate and lauryl sulphate. When the eggs were immersed in a 0.5 per cent solution of Monogen in sea water, the breakdown of the cortical granules began in a short time and membrane elevation was completed in 2 minutes at 18° C. Too long an exposure to the Monogen solution resulted in cytolysis of the eggs. However, if the eggs were washed with sea water immediately after the membrane was formed, they could be caused to develop into larvae after an appropriate treatment with a hypertonic solution. Thus, Monogen has proved to be an excellent parthenogenetic agent. Lipon, another detergent, consisting chiefly of alkyl sulfonate, has a similar effect. It was found that the result of partial exposure of the egg surface to Monogen or Lipon was almost the same as those with wasp-venom and sodium choleinate.

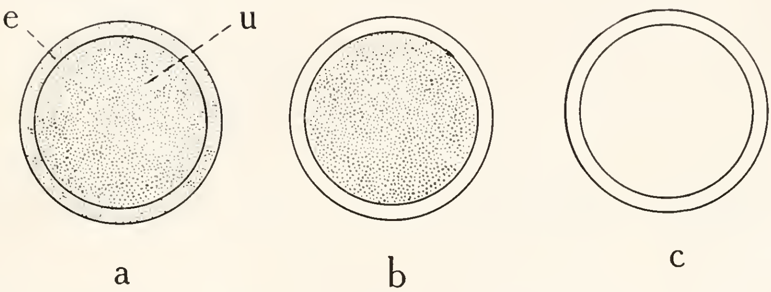


FIGURE 3. Diagrams to show the cortical response of eggs partially exposed to various reagents. a, unexposed egg. The cortical granules are intact all over the egg. b, an egg exposed to wasp-venom, sodium choleinate or Monogen. c, an egg exposed to fatty acid, non-electrolyte or distilled water. e, equatorial region; u, upper part of the egg adhering closely to glass surface.

These results suggest that the cortical change caused by treatment with wasp-venom, sodium choleinate, Monogen and Lipon is not of a propagating nature.

### *Experiments with fatty acids, non-electrolytes and distilled water*

Monobasic fatty acids are known to be very effective in inducing membrane formation in sea urchin eggs. Experiments were performed employing the technique described above, in order to determine whether the effect of fatty acids is of a propagating nature. The egg surface was partially exposed to butyric acid sea water (47 cc. of sea water plus 3 cc. of N/10 butyric acid) for 30 seconds at 18° C., and then the solution was replaced by normal sea water. After about 20 seconds the cortical granules in the equatorial region of the egg began to break down and a membrane started to separate. The granular breakdown then rapidly proceeded in the upper unexposed part of the egg and within 5 to 9 seconds their breakdown was finished all over the egg surface (Fig. 3c). Acetic and propionic acids also were found to have the same effect. It is apparent that these results are entirely different from those with wasp-venom, sodium choleinate and detergents. It is concluded that the effect of fatty acids is of a propagating nature.

It has been known since the publication of Motomura's work (1934) that urea solution is an excellent activating agent. Moser (1940) has observed that Arbacia eggs treated with molar concentrations of non-electrolyte solutions exhibit the same kind of visible cortical response as that obtained with sperm cells and other stimulating agents. Therefore, it is of interest to determine whether the effect of non-electrolytes is of a propagating nature. Molar solutions (pH 7.0) of urea, sucrose and glycerine were employed. Among these, the urea solution proved the most suitable, since almost 100% of the eggs could be activated. The experimental results were the same as those obtained with fatty acids, indicating that the effect of a non-electrolyte solution is of a propagating nature.

Another series of experiments was undertaken to study the effect of distilled water. It was found that distilled water also was a strong stimulating agent, having the same effect as that of fatty acids.

## Discussion

Up to the present, numerous reagents have been found to be effective for inducing the elevation of the fertilization membrane in sea urchin eggs. The range of such effective reagents is exceedingly wide, but in every case the first visible cortical response, regardless of the nature of the reagent, is the breakdown of the cortical granules (Moser, 1939). The elevation of the membrane follows this initial visible cortical response. On the basis of these facts it has been believed by some workers that stimulating reagents other than sperm cells effect essentially the same type of cortical response as that obtained upon insemination. However, the results presented in this paper make it possible to classify the stimulating reagents into two groups according to the nature of their effects. This further means that their effects are not always the same so far as the invisible cortical response is concerned. The first group includes reagents such as butyric acid, distilled water and isotonic solutions of non-electrolytes. The cortical change provoked by these reagents is of a propagating nature. To the second group belong reagents such as wasp-venom, sodium choleinate, Monogen and Lipon. The response to their effects is of a non-propagating nature.

It should be noted here that the propagating nature of the change induced by reagents which belong to the first group is capable of proof only when the egg surface is partially exposed to the reagents. In usual experiments the eggs in any solution are completely exposed to it, so that the propagating nature of the response is not detectable. The question arises, therefore, as to the occurrence of the propagation of the response in such cases. Yamamoto (1944) has shown in Oryzias that there is a gradient of irritability in the cortex of the unfertilized egg. According to him, the irritability is highest at the animal pole, medium at the equator and lowest at the vegetal pole. If it be assumed that there is a gradient of irritability in the cortex of the sea urchin egg also, and in addition that the response occurs first in the most irritable part of the cortex when the egg is immersed in a solution of a stimulating reagent, it would seem probable that the response might travel in a rapid wave-like fashion around the egg cortex before the direct response to the reagent occurred in the other part. At present, however, no evidence has been furnished as to such a gradient of irritability in the sea urchin egg. Therefore, there remains another possibility, that the cortical response in all parts of the egg surface takes place at the same time, being directly provoked by the chemical stimulus. However, regardless of this, the response induced by a reagent of the first group should be considered to be essentially different from that induced by reagents of the second group, since the former was able to propagate over a part of the egg surface not exposed to the reagent, while the latter was not.

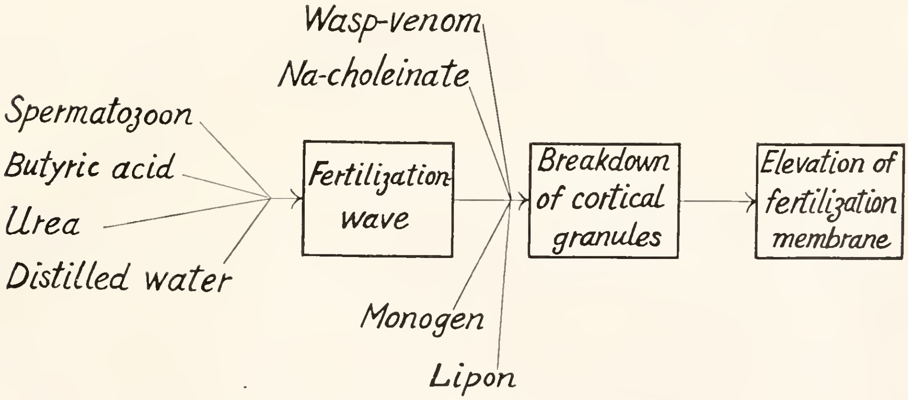


FIGURE 4. Diagram showing the process of the cortical change.

In the first paper of this series it was shown that the breakdown of one cortical granule does not automatically induce the breakdown of its neighbours. The author is therefore convinced that there must be an invisible wave-like change underlying the breakdown of the cortical granules whenever the cortical change is of a propagating nature. According to this concept, reagents such as urea or butyric acid stimulate the eggs to initiate an invisible wave-like change, which is followed by the breakdown of the cortical granules.

In the previous paper it was also postulated that some invisible cortical change of a propagating nature must occur at the time of fertilization. This invisible cortical change was called the "fertilization-wave." The nature of the cortical change provoked by the reagents of the first group discussed in this paper suggests that this change is essentially the same as that resulting from the entrance of spermatozoa. If this suggestion be true, then it may be said that the reagents of the first group induce the fertilization-wave itself, while the reagents of the second group induce the same changes as the fertilization-wave, without the intervention of the fertilization-wave. This conclusion is essentially in agreement with the view considered on the effect of wasp-venom and sodium choleinate in the previous paper.

These concepts give us the scheme diagrammed in Figure 4. It shows that the spermatozoon and the reagents of the first group give rise to the fertilization-wave and this causes the breakdown of the cortical granules. The reagents of the second group cause the breakdown of the cortical granules without the intervention of the fertilization-wave. The granular breakdown, then, is followed by the elevation of the fertilization membrane, regardless of the nature of the activating reagent.

In a recent paper, Runnström and Kriszat (1952) reported that in Psammechinus eggs the propagation of the impulse caused by the attachment of the spermatozoon is inhibited by the attachment of the egg surface to glass. The data on Hemicentrotus eggs do not agree with these findings. Either there is a great difference in these two sea urchins with respect to the nature of the cortex, or the dissimilarities must be accounted for by differences in the conditions under which the observations were made.

The author wishes to express his gratitude to Prof. T. Yamamoto, Dr. Jean C. Dan and Mr. E. Nakano for their valuable suggestions and advice concerning the experiments and the manuscript.

## Summary

1. A special technique has been developed for partial exposure of a sea urchin egg surface to stimulating reagents under microscopical observation.

2. The surfaces of Hemicentrotus eggs were partially exposed to stimulating reagents for an appropriate time and the unexposed surface was examined after washing with sea water.

3. When wasp-venom, sodium choleinate, Monogen or Lipon was used, the cortical granules in the exposed cortex were completely broken down within a few minutes, while those in the unexposed cortex remained quite intact, showing no sign of the influence of the reagent. The effect of these reagents is believed to be of a non-propagating nature.

4. When butyric acid, acetic acid, propionic acid, distilled water or an isotonic solution of a non-electrolyte was used, the granular breakdown proceeded rapidly in the unexposed part of the cortex immediately after granule breakdown in the exposed part. Therefore, it is concluded that these reagents induce a cortical change of a propagating nature.

5. It is suggested that the nature of the cortical change provoked by reagents of the latter group is essentially the same as that which follows the entrance of spermatozoa.

## LITERATURE CITED

Moser, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. II. Response to chemical and physical agents. *J. Exp. Zool.*, **80**: 447–471.

Moser, F., 1940. Studies on a cortical layer response to stimulating agents in the Arbacia egg. III. Response to non electrolytes. *Biol. Bull.*, **78**: 68–79.

MOTOMURA, I., 1934. On the mechanism of fertilization and development without membrane formation in the sea urchin egg, with notes on a new method of artificial parthenogenesis. *Sci. Rep. Tohoku Imp. Univ., B,* **9**: 33–45.

RUNNSTRÖM, J., AND G. KRISZAT, 1952. The cortical propagation of the activation impulse in the sea urchin egg. *Exp. Cell Res.,* **3**: 419–426.

SUGIYAMA, M., 1952. Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. I. Response to sodium choleinate and wasp-venom. *Biol. Bull.,* **104**: 210–215.

YAMAMOTO, T., 1944. Physiological studies on fertilization and activation of fish eggs. II. The conduction of the "fertilization-wave" in the egg of *Oryzias latipes. Ann. Zool. Jap.,* **22**: 109–136.

# PROLONGATION OF LIFE–SPAN OF SEA URCHIN SPERMATOZOA, AND IMPROVEMENT OF THE FERTILIZATION–REACTION, BY TREATMENT OF SPERMATOZOA AND EGGS WITH METAL–CHELATING AGENTS (AMINO ACIDS, VERSENE, DEDTC, OXINE, CUPRON)

ALBERT TYLER

*Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, Calif.*[1]

The addition of an amino acid to the sea water in which spermatozoa of sea urchins and other marine invertebrates are diluted has been found (Tyler, 1950; Tyler and Atkinson, 1950) to extend very considerably their functional life-span. By means of these agents the duration of fertilizable life and motility may be extended more than 50-fold. Treatment of the spermatozoa also has an effect on the nature of the fertilization reaction. Thus, eggs of Lytechinus often exhibit incomplete membrane elevation when fertilized by spermatozoa that have been diluted in sea water. In general this effect depends upon the extent of dilution and the age of the sperm suspensions. Treatment of the spermatozoa with an amino acid corrects this effect where it occurs with freshly diluted sperm and, parallel to the extension of fertilizable life and motility, extends the period during which a good fertilization-reaction is elicited. Runnström *et al.* (1946; *cf.*, Runnström, 1948; Wicklund and Gustafson, 1949) have reported that treatment of so-called underripe eggs of sea urchins with glycine and other amino acids and fertilization in these solutions improves their fertilizability and gives good membrane elevation. Tyler and Atkinson (1950) have also obtained this effect but note that pre-treatment of the eggs and inseminating in sea water gave much less improvement in fertilization and membrane elevation than obtained by treatment of the sperm.

In starfish another effect of amino acid treatment of the sperm was discovered by Metz and Donovan (1950). This consists in enabling the sperm to be agglutinated by the egg water of this species whereas ordinarily the agglutination reaction fails.

The extension of life-span of the sperm is accomplished with no apparent utilization of the amino acid. Thus determinations of glycine showed no significant loss during prolonged incubation with sperm, nor was there any significant production of ammonia (Tyler and Atkinson, 1950). Similar tests with carboxyl $C^{14}$-labelled glycine showed a negligible amount of decarboxylation (Tyler and Rothschild, 1951). The metabolism of the spermatozoa is, however, affected by the presence of the amino acid (Tyler and Rothschild, 1951). When sea urchin spermatozoa are diluted in ordinary sea water there is an initial great increase in rate of respiration followed by a decrease to barely measurable values accompanying loss of fertilizing

---

[1] Part of the work reported here was performed at the Marine Biological Laboratory, Woods Hole, Mass.

This work was supported in part by a grant from the John Simon Guggenheim Memorial Foundation.

capacity and motility. In the presence of amino acid the initial burst of oxygen uptake is suppressed, proportionately to the concentration of the amino acid. The rate then decreases to a relatively low but quite appreciable value where it remains rather constant during the extended life-span of the sperm. The total oxygen uptake of the treated sperm during its extended life span considerably exceeds that of the controls. From this, together with the evidence of non-utilization of the added amino acid it is clear that death of the sperm in ordinary sea water is not due to exhaustion of oxidizable substrate. The addition of amino acid evidently enables the spermatozoa to utilize more fully their endogenous substrate. Another interesting effect of the amino acid is to enable the spermatozoa to remain motile and retain fertilizing capacity under anaerobic conditions, whereas in ordinary sea water they die promptly in absence of oxygen.

In view of the above-mentioned evidence it was suggested (Tyler and Rothschild, 1951) that the amino acids act by virtue of their ability (*cf.*, Greenberg, 1951; Martell and Calvin, 1952) to bind certain heavy metals ordinarily present in sea water. This hypothesis is supported by the results of tests with other kinds of metal-chelating agents and with artificial sea water reported in the present paper.

## Material and Methods

The animals used in these experiments were: the sea urchins *Arbacia punctulata, Strongylocentrotus purpuratus* and *Lytechinus pictus;* the sand dollar *Echinarachnius parma* and the polychaet *Chaetopterus pergamentaceus.* The eggs and sperm were obtained from the sea urchins and the sand dollars by the KCl-injection method (Tyler, 1949). From Chaetopterus they were obtained by excision of parapodia containing the ripe gonads.

The sperm concentrations are expressed as a percentage (1% and 0.1% in most of the experiments) of undiluted semen, the suspensions being prepared by direct dilution of the "dry" semen in the test solutions or immediately after a preliminary 5-fold dilution in sea water. "Dry" semen of the sea urchins *S. purpuratus* and *L. pictus* contains approximately $2 \times 10^{10}$ spermatozoa per ml. The suspensions were kept at room temperature (19° to 22° C.) in Pyrex, 25 or 50 ml., stoppered Erlenmeyer flasks on a slow rocker (10 c.p.m. over an angle of 60°) or, in a few experiments, unshaken in a shallow (1 mm.) layer.

Fertilizing capacity was determined from the results of inseminating freshly collected eggs (ca. 500) in 4 ml. of sea water with various amounts (usually forming a 2-fold dilution series) of the sperm suspensions. The senescing experimental and control sperm suspensions are thus compared with each other, as well as with the freshly diluted semen, on the basis of the amount required to give the same percentage of fertilization, where this percentage is less than 100 and greater than 0. From this the relative duration of fertilizable life is obtained. Since fertilizing capacity tapers off gradually as the sperm suspensions approach the end of their life-span but shows a rather rapid decline around the 50 per cent value it was considered best to compare the suspensions on the basis of their half-life-spans. These are the figures given in Tables I and II. It should be noted, however, that on other bases of comparison such as "total" life-span or 10% life-span the results are qualitatively the same and the relative values do not differ very greatly.

For the motility determinations the intervals at which readings were taken were determined on the basis of preliminary tests so that the usually sharp drop-off could be reasonably adequately covered. Motility was scored as 0, 1, 5, 25, 50, 75, 90 and 100 per cent. Again the duration of motility is expressed (Tables I and II) as a half-life. "Total" life-spans generally range some 50 to 100 per cent greater.

Sea water solutions of the various test substances were prepared from stock solutions made up with glass double-distilled water and adjusted to the pH of the sea water. Osmotic pressures were adjusted so that the most concentrated solutions employed did not deviate by more than 5 per cent from isotonicity with sea water. The sea water in most of the experiments was collected outside the laboratory at incoming tide and was filtered through washed "sharkskin" paper. It should be noted that it is important to check the pH of each solution made up by dilution of a stock solution in sea water. Thus, for example, when a stock solution of ethylenediaminetetraacetic acid at pH 8.2 is diluted with sea water to make a $10^{-3}$ molar solution there is a drop in pH to about 6.2. This is due to the displacement of protons from the weakly acidic ammonium groups of this compound as a result of chelation with some of the cations present in the sea water (*cf.*, Martell and Calvin, 1952).

## EXPERIMENTS

*Life-span of spermatozoa in solutions of amino acids and peptides.* In Table I the results of experiments with ten different amino acids and two peptides are summarized. All of these proved to be effective in extending both the fertilizable life and duration of motility of the spermatozoa. For glycine the optimum concentrations range around 0.05 and 0.1 molar. For the others the data are insufficient to specify optimum concentrations. The cysteine concentrations refer only to the initial solutions since this substance is rapidly oxidized to cystine upon preparation of the suspensions. The indications are that this may be effective in lower concentration than is glycine and the same may be true for glutathione.

The degree of extension of life-span obtained with these agents is dependent on the density of the sperm suspension. As is well known (*cf.*, Gray, 1928a, 1928b, 1931; Rothschild, 1948, 1951) the life-span of spermatozoa decreases with increasing dilution of the suspension. Comparison of the values of half-life-span in sea water (column headed 0) for the different semen concentrations, in experiments employing the same species, illustrates this dilution effect. In the amino acid solutions the "absolute" extension of life-span, in hours, is generally greater for the denser than the more dilute suspensions. The relative increase in life-span is, however, greater the more dilute the sperm suspension. Thus, in the experiments with Lytechinus listed in Table I the average half-life-spans for the 5, 1, 0.5 and 0.1 per cent sperm suspensions in 0.05 $M$ glycine are, respectively, 7 to 12, 18 to 46, 50 to 80 and 100 times those for the sea water controls.

The present experiments also show a correlation between fertilizing capacity and motility. While it is well known (see Tyler, 1948; Rothschild, 1951 for references) that motile spermatozoa can be rendered non-fertilizing by various means, it is general experience that non-motile spermatozoa cannot effect fertilization. The present results indicate that loss of fertilizing capacity roughly parallels loss of motility.

*Life-span of spermatozoa in solutions of other metal-chelating agents.* In this category the effect of the following substances was investigated: ethylenediamine-

TABLE I

*Action of amino acids on life-span of spermatozoa at 19°–22° C.*

| Substance | Species | Semen conc. | No. of experiments | Half-motile life* and half-fertilizable life† in: (concentration in sea water) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 0.25 *M* | 0.1 *M* | 0.05 *M* | 0.025 *M* | 0.01 *M* | 0.001 *M* |
| | | | | hrs. | hrs. | hrs. | hrs. | hrs. | hrs. | hrs. |
| Glycine | *S. purp.* | 1% | 1* | 2 | 48 | 53 | 27 | 7 | 5 | 1 |
| | | 1% | 1† | 1 | 20 | 40 | 40 | 15 | 10 | 1 |
| | | 0.1% | 1* | 0.5 | 26 | 31 | 17 | 2.5 | 0.5 | 1 |
| | | 0.1% | 1† | 0.5 | 10 | 25 | 20 | 5 | 0.5 | 0.5 |
| | *L. pict.* | 5% | 3* | 1.5 | | | 18 | | | |
| | | 5% | 2† | 2 | | | 15 | | | |
| | | 1% | 3* | 1 | | | 18 | | 1 | 1 |
| | | 1% | 1† | 0.5 | | | 23 | | | |
| | | 0.5% | 8* | 0.2 | | | 16 | | | |
| | | 0.5% | 3† | 0.2 | | | 10 | | | |
| | | 0.1% | 2* | 0.02 | 1.3 | 3 | 2 | | 0.05 | 0.05 |
| | *A. punct.* | 1% | 1*† | 6–5 | | | 20 | | | |
| | | 0.5% | 1*† | 2–1 | | | 49+ | | | |
| | | 0.1% | 1*† | 0.5 | | | 4+ | | | |
| | | 0.05% | 2*† | <0.5 | | | 2.5+ | | | |
| | *E. parma* | 0.25% | 1*† | 1 | | | 8.5+ | | | |
| | *Ch. perg.* | 0.1% | 1*† | 0.3 | | | 4.5+ | | | |
| Alanine | *L. pict.* | 0.1% | 1*† | 0.02 | | 1+ | | | | |
| | *A. punct.* | 0.1% | 1*† | 1 | | 24+ | | | | |
| Valine | *L. pict.* | 0.1% | 1*† | 0.02 | | 1+ | | | 0.02 | |
| Leucine | *L. pict.* | 0.1% | 1*† | 0.02 | | 1+ | | | 0.02 | |
| Lysine | *L. pict.* | 0.1% | 1*† | 0.02 | | 1+ | | | 0.02 | |
| Glutamic | *L. pict.* | 0.5% | 1* | 0.1 | | | 20+ | | | |
| | *A. punct.* | 0.1% | 1*† | 5 | | | 40+ | | | |
| Histidine | *A. punct.* | 0.5% | 1*† | 4 | | | 10+ | | | |
| | *A. punct.* | 0.1% | 1*† | 1.5 | | | 10+ | | | |
| | *A. punct.* | 0.01% | 1*† | 0.02 | | | 10+ | | | |
| Phenylalanine | *A. punct.* | 0.02% | 1*† | 0.02 | | | 3.5+ | | | |
| | *A. punct.* | 0.005% | 1*† | 0.02 | | | 3.5+ | | | |
| Tryptophane | *A. punct.* | 0.02% | 1*† | 0.02 | | | 3.5+ | | | |
| | *A. punct.* | 0.005% | 1*† | 0.02 | | | 3.5+ | | | |
| Cysteine | *A. punct.* | 0.05% | 1*† | 2 | | | 1 | | 10+ | 10+ |
| | *A. punct.* | 0.01% | 1*† | 0.5 | | | 1 | | 2 | 4 |
| | *A. punct.* | 0.002% | 1*† | <0.02 | | | 0.5 | | 2 | 2 |
| Glycylglycine | *A. punct.* | 0.5% | 1*† | 6 | | | 48+ | | | |
| Glutathione | *L. pict.* | 1% | 1* | <0.4 | | 3 | 2 | 2 | 1.4 | 0.5 |
| | *L. pict.* | 0.1% | 1* | <0.3 | | 1.8 | 2.5 | 1 | 1 | 0.3 |

tetraacetic acid (known commercially as Versene[2]), diethyldithiocarbamic acid (DEDTC), 8-hydroxyquinoline (oxine), α-benzoinoxime (cupron), N-nitrosophenylhydroxylamine (cupferron), diazoaminobenzene, p-aminophenol, o-nitrophenol, 1–3, 4-trihydroxychalcone, p, p′-methylene bis N,N-dimethylaniline and 2-hydroxy-3-methoxybenzaldehyde. Only the first four of these were effective in extending the life-span of the sperm. However, since the others were each tested in only one set of experiments there is the possibility that some of these might prove effective on further investigation.

---

[2] I am indebted to the Bersworth Chemical Company, Framingham, Massachusetts, for samples of this substance; and to Dr. Linus Pauling for suggesting its use in connection with these experiments.

The results of experiments with the first four substances are summarized in Table II. Of these, Versene and DEDTC have given the longest extensions of life-span. Versene proved to be highly effective at concentrations from $10^{-3}$ to $10^{-5}$ molar and DEDTC at concentrations of $10^{-3}$ and $10^{-4}$. Two experiments not listed in the table also show DEDTC to be effective at $10^{-5}$ molar (7 hours half-motile life vs. 1½ hours for the sea water control with 1% Lytechinus sperm). Both substances were inhibitory at $10^{-2}$ molar.

Table II

*Action of various metal-chelating agents on life-span of sea urchin spermatozoa at 19°–22° C.*

| Substance | Species | Semen conc. | No. of experiments | Half-motile life* and half-fertilizable life† in: (concentration in sea water) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | $10^{-2}$ *M* | $10^{-3}$ *M* | $10^{-4}$ *M* | $10^{-5}$ *M* | $10^{-6}$ *M* | $10^{-7}$ *M* |
| | | | | hrs. | hrs. | hrs. | hrs. | hrs. | hrs. | hrs. |
| Versene[1] | *S. purp.* | 1% | 8* | 2.9 | 0.3 | 21 | 22 | 13 | 7 | 7 |
| | *S. purp.* | 1% | 3† | 1 | <1 | 20+ | 20+ | 10–15 | | |
| | *S. purp.* | 0.1% | 4* | 0.2 | <0.1 | 3 | 10 | 1.5 | 0.3 | 0.2 |
| | *L. pict.* | 1% | 4* | 3.2 | | 21 | 27 | 29 | | |
| | *L. pict.* | 1% | 2† | 0.8 | | 24+ | 24+ | 24+ | | |
| | *L. pict.* | 0.1% | 1* | 0.2 | | 18 | 22 | 13 | | |
| DEDTC[2] | *S. purp.* | 1% | 3* | 5.6 | 2.5 | 17 | 5.5 | | | |
| | *L. pict.* | 1% | 8* | 5.8 | 2.7 | 22 | 32 | | | |
| | *L. pict.* | 1% | 2† | 1 | | | 30+ | | | |
| | *L. pict.* | 0.1% | 1* | <0.5 | 1 | 4 | 2.5 | | | |
| Oxine[3] | *S. purp.* | 1% | 3* | 2 | | 16 | 14 | 2 | | |
| | *S. purp.* | 0.1% | 1* | 0.1 | | 2 | 2 | 0.1 | | |
| | *L. pict.* | 1% | 2* | 0.4 | | 4.5 | | 1.5 | 0.8 | 0.5 |
| Cupron[4] | *S. purp.* | 1% | 2* | 1 | | | 6 | 8.5 | 5 | |
| | *S. purp.* | 0.1% | 2* | 0.3 | | | 2 | 2 | 0.3 | |

[1] Ethylenediaminetetraacetic acid.
[2] Diethyldithiocarbamate.
[3] 8-Hydroxyquinoline.
[4] α-Benzoinoxime.

The degree of extension of life-span obtained with these agents is of the same order as was obtained by use of the amino acids. It seems reasonable to conclude, then, that they are acting in similar manner, namely by virtue of their metal-chelating capacity. Differences in effective range of concentrations can be attributed to differences in the dissociation constants of the metal-chelate compounds. Martell and Calvin (1952) have assembled data on the stability constants (reciprocal of dissociation constants) for an extensive series of metal-chelate compounds, including most of the agents used in this work. While the data on optimum concentrations accumulated here do not permit detailed quantitative comparisons, qualitatively the differences between the amino acid glycine and the other chelating agents, such as Versene, correspond to the differences in their relative avidity for metal ions.

*Life-span of spermatozoa in artificial sea water with various amounts of calcium.* In order to examine further the view that the extension of life-span by the amino acids and other metal-chelating agents was occasioned by the binding of certain metal ions, tests were made with artificial sea water. This was prepared from Merck Reagent Grade chemicals, of which the NaCl was especially low ($< 0.0001\%$) in heavy metals. Based on the sea water analyses of Lyman and Fleming (1940) the artificial sea water was made up with the following composition: 1000 ml. 0.55 $M$ NaCl, 22 ml. 0.55 $M$ KCl, 195 ml. 0.37 $M$ $MgCl_2$, 103 ml. 0.37 $M$ $Na_2SO_4$, 6 ml. 0.55 $M$ $NaHCO_3$ and 35 ml. 0.37 $M$ $CaCl_2$, adjusted to pH 8.2.

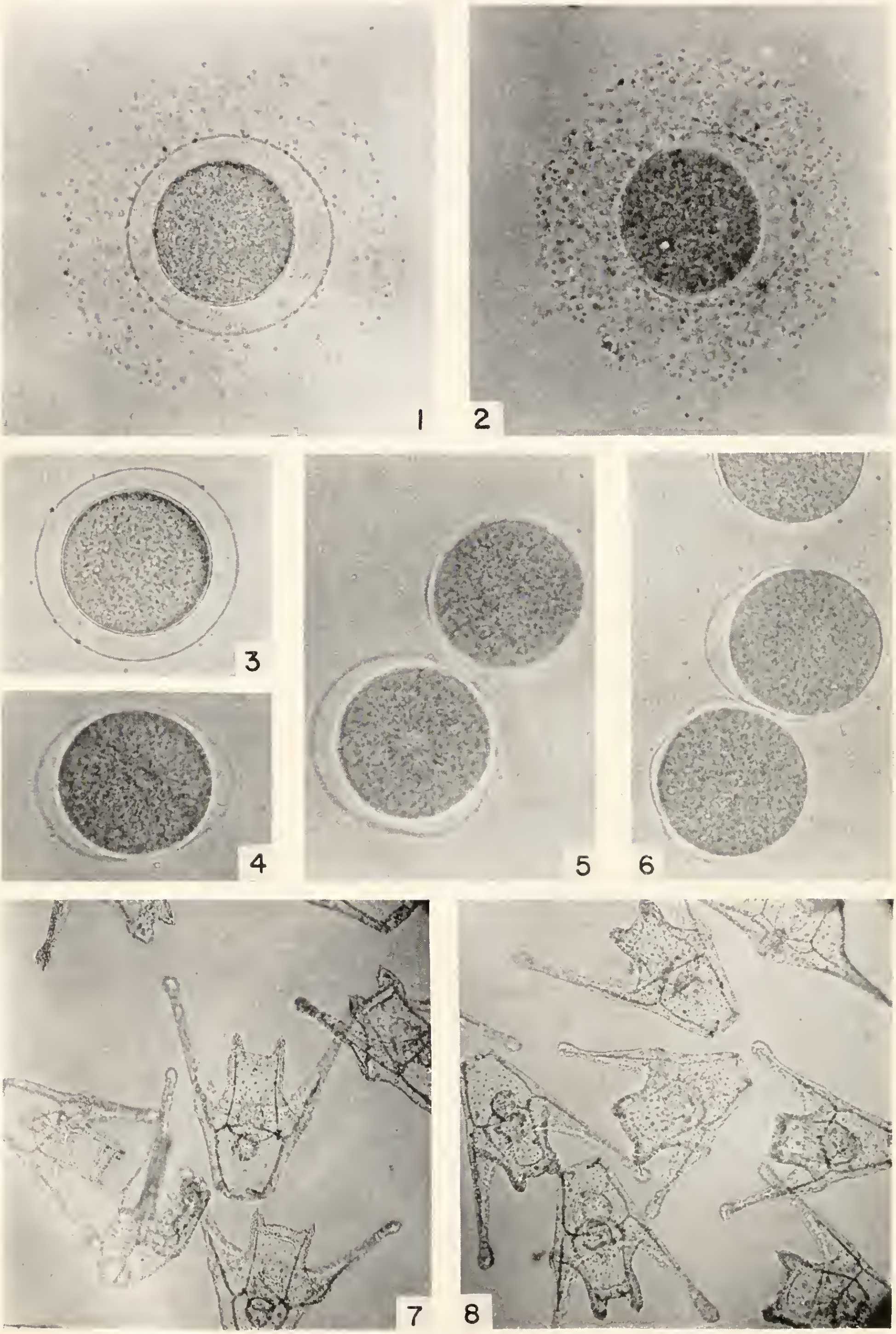
Along with the tests of the artificial sea water the effect of varying the concentration of calcium was also examined, using the above formula with various amounts of the $CaCl_2$ solution. Eight sets of determinations were made of the duration of motility of sperm of Strongylocentrotus in 1% and 0.1% suspension in ordinary sea water and in the artificial sea waters in which the calcium concentration ranged from 0 to $8 \times 10^{-2}$ molar. The average half-life-spans, in hours, in these experiments were:

| | Ordinary sea water | Artificial sea water containing calcium at molar concentration | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.0006 | 0.0025 | 0.01 | 0.02 | 0.04 | 0.08 |
| 1% sperm | 3.7 | 15.1 | 14.6 | 15.3 | 16.1 | 11.3 | 12.4 | 8.2 |
| 0.1% sperm | 0.7 | 3.6 | 4.1 | 3.3 | 6.7 | 0.8 | 1.6 | 1.4 |

As the figures show, the spermatozoa survived considerably longer in most of the artificial solutions than in ordinary sea water. Ordinary sea water is about 0.01 molar in calcium. The artificial sea water with this concentration of calcium gave the longest average life-span for the 0.1% sperm suspension but with the 1% suspension it did not differ appreciably from those with lower concentrations. Even in the "Ca-free" solution the sperm were found to survive longer than in ordinary sea water. This solution is, of course, not actually Ca-free since the added semen contributes calcium, which would probably amount to about 0.0001 molar for the 1% suspension and 0.00001 molar for the 0.1%.

It appears, then, that a balanced salt solution made from chemicals relatively low in heavy metals enables the sperm to survive longer than does natural sea water. Since this effect is obtained with solutions in which the concentration of calcium varies over a very wide range it is clear that this ion is not primarily concerned in the results obtained by use of the metal-chelating agents. Thus the action of Versene in extending the life-span of the sperm is not simply attributable to its known ability to bind calcium. While this chelating agent forms complexes with other alkaline earth metals the fact that the amino acids such as glycine, which form relatively weak complexes with these ions, are effective would tend to rule them out as being primarily involved in the life-span extending effect. It seems most reasonable to conclude that heavy metals are involved, although the present evidence does not permit ready identification of these. Rothschild and Tuft (1950) found that the dilution effect of sea water on rate of oxygen uptake could be imitated by trace amounts of $CuCl_2$ or $ZnCl_2$ dissolved in isotonic 'Analar' (containing negligible

PLATE I. Eggs and embryos of *Lytechinus pictus.* Magnifications: 175 × for Figures 1 to 6 and 65 × for Figures 7–8.



amounts of heavy metal) NaCl. The isotonic 'Analar' NaCl itself does not give a dilution effect when added to the sperm suspension. Glycine solutions can also abolish the dilution effect (Tyler and Rothschild, 1951). On the basis of this evidence it is suggested that $Cu^{++}$ and $Zn^{++}$ are among the heavy metal ions whose removal is responsible for the life-span extending effect of the chelating agents.

*Effect of treatment of the sperm on membrane-elevation.* As was mentioned earlier, treatment of the sperm with solutions of the amino acids improves the fertilization-reaction induced upon insemination with dilute and with aged sperm suspensions. The same effect is obtained with other chelating agents. In Plate I examples of good and poor membrane-elevation of eggs of Lytechinus are shown. The effect on the fertilization-reaction is illustrated by the results of the experiments with glycine and with Versene presented in Table 3. It may be noted that with sperm diluted in ordinary sea water, and aged only a short time, the type of reaction, along with the percentage fertilization, improves upon insemination with increasing amounts of sperm. However, with sperm that have been diluted in glycine or in Versene, and similarly aged, much smaller amounts suffice to give 100 per cent fertilization and normal membrane-elevation. Upon aging, the rapid drop-off in fertilizing capacity is seen along with the poor fertilization-reaction induced by the sea water-sperm. The glycine- or Versene-treated sperm, upon aging, maintain not only their fertilizing capacity but also their ability to induce good membrane-elevation. Towards the end of their extended life-span they, too, may induce poor membrane-elevation when used in amounts that were earlier effective in inducing a good reaction and 100 per cent fertilization. The type of reaction given by the eggs is evidently dependent upon the amount of sperm used for insemination as well as the age of the suspension. This holds both for the sea water-sperm and for the glycine- or Versene-treated sperm. Excessive amounts of sperm may sometimes be inhibitory, as the figures in Table VII indicate. Occasionally, also, sea water-sperm fail to give good membrane-elevation with any amount tested while the glycine- or Versene-treated sperm induce normal membrane-elevation.

It appears from these results that the condition of the inseminating sperm can determine the type of fertilization-reaction given by the eggs. As the spermatozoa age in ordinary sea water an increasing proportion of them become impaired in such a way that, while still capable of fertilization, they cannot elicit a normal response on the part of the egg. The relatively poorer response elicited by the smaller

---

FIGURE 1. Good membrane-elevation shown by egg with intact jelly-coat inseminated with sperm treated with Versene ($10^{-3}$ molar in sea-water, ½ hour).

FIGURE 2. Poor membrane-elevation shown by egg with intact jelly-coat inseminated with about 8 times as much sea water-treated sperm (note larger number of sperm in the jelly-coat than in that of Figure 1).

FIGURE 3. Good membrane-elevation shown by jellyless egg inseminated with Versene ($10^{-3}$ $M$)-treated sperm.

FIGURE 4. Poor membrane-elevation shown by jellyless egg inseminated with very small amount of Versene ($10^{-3}$ $M$)-treated sperm.

FIGURES 5 AND 6. Various types of poor membrane-elevation shown by Versene ($10^{-3}$ $M$)-treated and sea water-washed eggs inseminated with sea water-treated sperm.

FIGURES 7 AND 8. Five day old plutei (not fed) from eggs fertilized and allowed to develop in sea water (Fig. 7) and in $10^{-3}$ molar Versene in sea water (Fig. 8).

amounts of inseminate can be interpreted on the basis of their greater dilution in the insemination dishes and consequent increase in proportion of impaired sperm in the interval before fertilization. The poor response given on occasion with excess sea water-sperm can be attributed to an increased probability of the impaired sperm, present in high proportion in such suspension, encountering the eggs; whereas with somewhat lower amounts of inseminate the more motile and presumably unimpaired sperm would have the advantage. Whether or not other factors, such as the liberation of antifertilizin from the sperm (see Tyler and Atkinson, 1950), might also be involved, would need to be further investigated.

TABLE III

*Fertilization-reaction of eggs of Lytechinus inseminated with various amounts of sperm that have aged in sea water (s.w.), 0.05 M glycine (gl) and 0.001 M Versene (ve)*

| Age of 1% sperm susp. | Solution | Percentage of good membrane-elevation (G) and poor membrane-elevation (P) upon insemination of ca. 500 eggs in 4 ml. of sea water with following volumes of 1% sperm aged as indicated | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 ml. | | 0.10 ml. | | 0.2 ml. | | 0.4 ml. | |
| | | G | P | G | P | G | P | G | P |
| (hours) | | | | | | | | | |
| 0.02 | s.w. | 10 | 80 | 25 | 75 | 100 | 0 | 100 | 0 |
| | gl. | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 0.5 | s.w. | 1 | 20 | 0 | 20 | 10 | 75 | 95 | 5 |
| | gl. | 100 | 0 | 98 | 0 | 100 | 0 | 100 | 0 |
| 1, 2, 5 and 10 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | gl. | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 18 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | gl. | 90 | 5 | 100 | 0 | 95 | 5 | 100 | 0 |
| 23 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | gl. | 0 | 15 | 10 | 30 | 35 | 50 | 85 | 15 |
| 0.2 | s.w. | 10 | 75 | 90 | 10 | 100 | 0 | 100 | 0 |
| | ve. | 100 | 0 | 100 | 0 | 90 | 10 | 100 | 10 |
| 1 | s.w. | 0.2 | 0 | 10 | 20 | 20 | 60 | 90 | 10 |
| | ve. | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 2 and 4 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ve. | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 10 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ve. | 15 | 3 | 95 | 5 | 100 | 0 | 100 | 0 |
| 21 | s.w. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ve. | 30 | 5 | 45 | 10 | 85 | 5 | 100 | 0 |

*Fertilization-reaction of Versene-treated eggs in Versene and in sea water.* As was noted above (see introduction), treatment of sea urchin eggs with glycine, and fertilization in the solution, improves their fertilizability and type of reaction. This occurs also with other chelating agents. Table IV gives the results of some experiments with Versene. Comparison of the last three lines of the table with the first line shows the great improvement in percentage fertilization and in membrane-elevation that is obtained upon insemination in the Versene solutions. The sperm used in this experiment were freshly diluted in sea water but, of course,

TABLE IV

*Fertilization-reaction of Versene-treated eggs of Lytechinus inseminated in sea water and in Versene*

| Eggs treated for ½ hour with | 0.1 ml. of egg-suspension transferred to 4 ml. of | Percentage good membranes (G) and poor membranes (P) upon insemination with following volumes of 1% sperm | | | |
|---|---|---|---|---|---|
| | | 0.05 ml. | | 0.20 ml. | |
| | | G | P | G | P |
| Sea water | Sea water | 0 | 30 | 5 | 75 |
| $10^{-5}$ *M* Versene | Sea water | 70 | 30 | 80 | 20 |
| $10^{-4}$ *M* Versene | Sea water | 60 | 40 | 100 | 0 |
| $10^{-3}$ *M* Versene | Sea water | 100 | 0 | 100 | 0 |
| $10^{-5}$ *M* Versene | $10^{-5}$ *M* Versene | 100 | 0 | 100 | 0 |
| $10^{-4}$ *M* Versene | $10^{-4}$ *M* Versene | 100 | 0 | 100 | 0 |
| $10^{-3}$ *M* Versene | $10^{-3}$ *M* Versene | 100 | 0 | 100 | 0 |

upon insemination of the eggs in the Versene solutions they are exposed to the action of this agent. So this type of experiment does not permit one to decide whether the effect is on the egg or sperm or both.

If the eggs are inseminated in sea water after treatment with Versene (lines 2, 3, and 4 of Table IV) there is much less improvement in the fertilization-reaction. In these tests insemination was done immediately after transfer to the sea water, and without further washing. It seemed likely that there might be sufficient carry-over of the Versene, which would perhaps diffuse only slowly from the gelatinous coat of the egg, to affect the results. Tests were therefore made of the effect of washing the eggs, after Versene-treatment, by repeated transfer in sea water or in Versene. The results of such a test are given in Table V. As the figures show, successive washing in sea water decreases the fertilizability of the Versene-treated eggs while those correspondingly transferred through Versene solution maintain their high fertilizability and ability to elevate good membranes.

*Effect of removal of jelly-coat.* It has been observed that the jelly-coat of eggs of various sea urchins swells considerably in solutions of amino acids and of proteins (Runnström *et al.*, 1943, 1946). This effect is readily observed, too, in Versene and DEDTC solutions. Thus, in $10^{-3}$ *M* Versene the jelly-coat of Lytechinus eggs swells within a half-hour to about double its original thickness.

TABLE V

*Effect of washing in sea water on fertilization-reaction of Versene-treated eggs of Lytechinus*

| Eggs treated 2 hrs. in $10^{-3}$ *M* Versene and washed in: | Percentage good membranes (G) and poor membranes (P) upon insemination with 0.05 ml. of 1% sperm. Eggs washed by transferring 0.1 ml. to 4 ml. of new solution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First washing | | Second washing | | Third washing | | Fourth washing | |
| | G | P | G | P | G | P | G | P |
| Sea water | 25 | 75 | 0 | 50 | 0 | 30 | 0 | 5 |
| $10^{-3}$ *M* Versene | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |

Concerning the action of the amino acids and proteins on the egg Runnström (1949, p. 251) points out that "This may facilitate penetration of spermatozoa and elevation of the fertilization membrane. But this is not the whole explanation of their improving effect." Eggs deprived of their jelly-coat when inseminated in these solutions also showed improved fertilization and membrane-elevation (*cf.*, Wicklund and Gustafson, 1949). It is suggested (Runnström, 1948) that these substances overcome a "cytoplasmic underripeness" of the egg. As the present work shows and was earlier reported (Tyler and Atkinson, 1950), treatment of the sperm alone with glycine, Versene, etc. induces good membrane-elevation. Treatment of the sperm with albumin (Wicklund, 1949) also has this effect. Also, increasing the amount of sea water-sperm used for insemination can result in improved membrane-elevation. It is not easy to see how a "cytoplasmic underripeness" can be involved in this.

In order to examine these questions further the reaction of jellyless eggs was investigated. The results of an experiment with Lytechinus eggs are given in Table VI. The eggs had stood for ½ hour in $10^{-3}$ molar Versene (III) and in sea water (II) and were then shaken to remove their jelly-coat. That the jelly-coat was effectively removed was checked by microscopic examination. They were then washed in several changes of sea water and aliquots inseminated with various amounts of a freshly diluted sea water-sperm suspension along with aliquots of similarly washed sea water-eggs with intact jelly-coat (I). Comparison of II with III in this table shows that the pretreatment with Versene occasioned no significant improvement in the fertilization-reaction or in the percentage fertilization obtained with a given amount of sperm. The eggs with intact jelly-coat (I) showed better fertilization with corresponding amounts of sperm, and this is consistent with earlier experiments (Tyler, 1941; Runnström, 1947) along this line. Similar results have been obtained with eggs pretreated with glycine and inseminated in sea water after removal of the jelly-coat. Also pretreatment of jellyless eggs with glycine or with Versene and insemination in sea water did not improve fertilization in comparison with the sea water-exposed jellyless eggs.

TABLE VI

*Effect of removal of jelly-coat on fertilization-reaction of eggs of Lytechinus*

| Volume of freshly diluted 1% sperm used for insemination | Percentage good membranes (G) and poor membranes (P) upon insemination in sea water after similar washing in sea water: | | | | | |
|---|---|---|---|---|---|---|
| | I. Eggs from sea water suspension with intact jelly-coat | | II. Eggs from (I) shaken to remove jelly-coat | | III. Eggs treated ½ hour in $10^{-3}$ *M* Versene, shaken to remove jelly-coat | |
| | G | P | G | P | G | P |
| 0.4 ml. | 100 | 0 | 90 | 10 | 100 | 0 |
| 0.2 ml. | 100 | 0 | 60 | 30 | 60 | 40 |
| 0.1 ml. | 90 | 10 | 20 | 20 | 10 | 50 |
| 0.05 ml. | 10 | 80 | 10 | 5 | 10 | 15 |
| 0.025 ml. | 5 | 45 | 0.5 | 0.5 | 3 | 2 |
| 0.013 ml. | 0 | 5 | 0.2 | 0.2 | 1 | 0.5 |

Since the action of these agents does not persist when the eggs are returned to sea water it does not seem likely that a "cytoplasmic maturation," in whatever sense Runnström (1948) may mean this, can account for the results. On the other hand treatment of the sperm and insemination of jellyless eggs in sea water results in improved fertilization, as it does in eggs with intact jelly-coat (Table III). Three such experiments are listed in Table VII. The eggs were pretreated with Versene in order to facilitate removal of the jelly-coat, washed and inseminated in sea water with various amounts of sperm that had been diluted and aged briefly in $10^{-3}$ *M* Versene or in sea water. Comparison of the Versene-treated with the sea water-sperm shows the former to have some 8 to 16 times the fertilizing capacity of the latter, and to induce good membrane-elevation when amounts of sperm are used that, in the case of the sea water controls, give mostly a poor fertilization-reaction. In both, the effect of quantity of sperm on type of reaction is shown, but with the Versene-treated sperm good membrane-elevation is given by considerably less sperm. This was illustrated also in the experiments on eggs with intact jelly-coat (Table III). An additional feature is observed in the results of the experiments of Table VII. With large amounts of sea water-sperm (0.8 ml. for experiment I; 0.8 and 0.4 ml. for experiments II and III) the fertilization-reaction is poorer than with the next lower amounts. This "optimum sperm concentration" effect is not regularly encountered in other experiments and presumably may be a property of particular sperm suspensions. An interpretation of this is offered in a preceding section. It may be concluded from the experiments with jellyless eggs that the improved fertilization-reaction induced in eggs with intact jelly-coat by

TABLE VII

*Fertilization-reaction of jellyless eggs of Lytechinus inseminated with various amounts of Versene-treated and untreated sperm*

| Volume of 1% sperm used for insemination | Percentage good membranes (G) and poor membranes (P). Eggs treated for ½ to 1 hour in $10^{-3}$ *M* Versene, shaken to remove jelly-coat, transferred to sea water (4 ml.) and inseminated with indicated volumes of sperm at 10 to 15 minutes after dilution in: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (Experiment I) | | | | (Experiment II) | | | | (Experiment III) | | | |
| | Sea water | | $10^{-3}$ *M* Vers. | | Sea water | | $10^{-3}$ *M* Vers. | | Sea water | | $10^{-3}$ *M* Vers. | |
| | G | P | G | P | G | P | G | P | G | P | G | P |
| ml. | | | | | | | | | | | | |
| 0.8 | 80 | 20 | 100 | 0 | 0 | 100 | 100 | 0 | 0 | 100 | 100 | 0 |
| 0.4 | 100 | 0 | 100 | 0 | 20 | 80 | 100 | 0 | 5 | 95 | 100 | 0 |
| 0.2 | 95 | 5 | 90 | 10 | 60 | 30 | 80 | 20 | 60 | 40 | 100 | 0 |
| 0.1 | 40 | 60 | 99 | 1 | 20 | 20 | 100 | 0 | 10 | 50 | 40 | 60 |
| 0.05 | 5 | 90 | 97 | 3 | 10 | 5 | 100 | 0 | 10 | 15 | 90 | 10 |
| 0.025 | 5 | 15 | 99 | 1 | 0.5 | 0.5 | 98 | 2 | 3 | 2 | 60 | 40 |
| 0.0125 | 2 | 3 | 97 | 3 | 0.2 | 0.2 | 45 | 50 | 1 | 0.5 | 10 | 85 |
| 0.0063 | 0 | 0 | 20 | 40 | 0 | 0 | 20 | 5 | 0 | 0 | 30 | 20 |
| 0.0031 | 0 | 0 | 20 | 10 | 0 | 0 | 45 | 5 | 0 | 0 | 20 | 5 |
| 0.0016 | 0 | 0 | 8 | 2 | | | | | | | | |
| 0.0008 | 0 | 0 | 0 | 0 | | | | | | | | |

treated sperm is not primarily due to an effect of such sperm on the jelly-coat or to greater ease of penetration of the jelly-coat.

*Development in glycine and in Versene solutions.* Glycine, in rather low concentration, interferes with development. Thus in a test with Lytechinus eggs, fertilized and allowed to remain in $10^{-3}$ molar glycine, development did not proceed beyond the formation of abnormal gastrulae. In stronger solutions stereoblastulae were generally formed. While fertilization and cleavage are obtained in solutions as strong as 0.125 molar, development generally stops in the early blastula and disintegration soon sets in. Transfer to sea water soon after fertilization permits normal development, as Wicklund and Gustafson (1949) found with weaker solutions. In Versene solutions development of Lytechinus eggs was found to proceed quite normally in concentrations up to 0.001 molar. In one test, for example, eggs inseminated in $10^{-3}$, $10^{-4}$ and $10^{-5}$ molar Versene gave 100 per cent fertilization and practically all eggs developed normally to the pluteus stage. Five-day old plutei from the $10^{-3}$ molar Versene solution and the sea water control are shown in Figures 7 and 8. Also eggs of Lychechinus that have been fertilized by sperm that have aged up to 24 hours in $10^{-3}$ molar Versene were found to develop normally to the pluteus stage.

## Discussion

Most of the points raised by the present experiments have already been discussed above, but a few may be further emphasized here.

The ability of the metal-chelating agents, Versene, DEDTC, oxine and cupron, and of artificial sea water of low metal content, to extend the life-span of spermatozoa provides strong support for the previously expressed view (Tyler and Rothschild, 1951) that the trace metals normally present in sea water are responsible for the usual early death of the sperm in this medium. The life-span extending action of the amino acids and peptides (Tyler, 1950; Tyler and Atkinson, 1950) is explainable on this basis as is also the action of protein (Metz, 1945; Wicklund, 1949) and seminal fluid (Hayashi, 1945). In birds and mammals, amino acids, protein and seminal fluid may be similarly involved (Lorenz and Tyler, 1951; Tyler and Tanabe, 1952; Chang, 1947, 1949).

Very likely seminal fluid owes its action largely to its proteins. This can help explain the well-known *Dilution Effect; i.e.,* the decrease in life span with increasing dilution of the semen in sea water or physiological salt solutions. Thus, in the denser suspensions more seminal fluid protein would be available to bind the heavy metals present and eliminate their toxic action. If this were the whole explanation of the dilution effect then in the presence of the proper concentration of metal-chelating agent the dilute suspensions should survive as long as the denser ones. While this has been approached in some experiments reported here, in most cases the dilute suspensions have not lasted as long. So, the question remains open as to what extent other factors (see Gray, 1928a, 1928b, 1931; Rothschild, 1951) may be involved. However, it is now clear (*cf.,* Tyler and Rothschild, 1951) that the early death of sperm diluted in ordinary sea water is not due to exhaustion of endogenous food reserves.

Identification of the particular heavy metals that may be involved in the toxic action of ordinary sea water on sperm is not readily feasible from the present results. As mentioned above there are good reasons for suspecting $Cu^{++}$ and $Zn^{++}$, but it is quite possible that others may also be concerned.

Of special interest is the ability of spermatozoa treated with amino acids and other chelating agents to improve the fertilization-reaction of the eggs. It is evident that the type of response given by the egg is not simply dependent upon the condition of the egg itself. The spermatozoon does not act in all-or-none manner in the sense of operating, or failing to operate, a trigger mechanism. Dependent upon its own condition the spermatozoon can elicit good or poor membrane-elevation on the part of the egg. This is not simply a matter of the treated sperm being able to traverse, and perhaps soften, the jelly-coat of the egg more effectively, since the effect is manifest also with jellyless eggs (Table VII).

In regard to possible effects on the egg the present experiments tend to rule out any maturing action. While insemination in Versene or glycine solutions improves fertilization, it is clear that this can be largely due to the effect on the sperm. Since pretreatment of the eggs with Versene or glycine and subsequent washing, and insemination in sea water, give no marked improvement in fertilization it appears that if there is any effect on the egg it is readily reversed. These substances cannot be considered to overcome any supposed "cytoplasmic underripeness" (Runnström, 1948, 1949) of the egg.[3] There are evidently effects of these substances on the eggs as manifest by swelling of the jelly coat and apparently also changes in the egg cytoplasm or its surface (*cf.*, Runnström and Monné, 1945; Runnström, 1948). However if these changes have any action in the direction of improved fertilizability of the egg it is evident that such effect largely disappears upon return to ordinary sea water, and it could hardly be considered a "maturating" effect. One might attempt to assess a possible fertilization-favoring effect on the egg by comparison of the results of inseminating also with treated-sperm eggs in sea water and in a solution of chelating agent. However, it would be difficult to decide to what extent the results are influenced by the effect on the sperm of the two different media in the insemination dishes. Runnström and his co-workers have contributed much to our knowledge of fertilization. While their extensive studies on "underripe" and "overripe" eggs do not represent a major part of their contributions it would appear desirable to re-examine the application of these terms to eggs. When difficulties in fertilization are encountered it would be important in the first place to know to what extent this is due to some agent ordinarily present in the sea water. Certainly where treatment of the sperm alone can improve the fertilization-reaction it does not seem reasonable to assume an "underripe" or "overripe" condition of the egg.

I am greatly indebted to Dr. T. Y. Tanabe, now at the Pennsylvania State College, for his help in this work, and also to Mrs. Joan Merritt for her participation in some of the later phases.

---

[3] While this manuscript was in preparation Dr. Hans Borei sent the author a copy of a note which he had prepared for publication in Experimental Cell Research. He reports that insemination in Versene solutions improves the fertilizability of underripe and overripe eggs of *Psammechinus miliaris*. Since pretreatment and similar tests were not performed he is undecided as to whether the increased fertilizability is due to effects on the eggs, the sperm, or both.

## SUMMARY

1. The duration of motility and of fertilizing capacity of sea urchin sperm diluted in sea water can be considerably extended by the addition of any one of certain metal-chelating agents.These include ethylenediaminetetraacetate (Versene), diethyldithiocarbamate, 8-hydroxyquinoline, α-benzoinoxime and various amino acids and peptides (glycine, alanine, valine, leucine, lysine, glutamic acid, histidine, phenylalanine, tryptophane, cysteine, glycylglycine, glutathione). The relative increase in life-span, in the presence of these agents, is greater the more dilute the sperm suspension, while the absolute increase is generally greater for the more concentrated suspensions. Over 100-fold extension of life-span of dilute suspensions has been obtained by use of these agents.

2. An artificial sea water of low heavy metal content also enables the sperm to survive longer than in ordinary sea water. This effect is obtained with artificial solutions in which the calcium concentration ranges from 8 times to 1/100 that of sea water.

3. The results support the previously suggested view that the increased survival of the sperm in presence of amino acids, proteins, etc. is due to the ability of these agents to bind heavy metals present in the dilution medium. It is suggested that $Cu^{++}$ and $Zn^{++}$ are among the metals involved.

4. The "Dilution Effect" (decreasing life-span with increasing dilution of suspension) is largely explained on the basis of similar action of the seminal fluid proteins which are present in higher concentration in the denser suspensions prepared by direct dilution of the semen.

5. Treatment of the sperm with glycine or with Versene improves the fertilization-reaction (membrane-elevation) induced upon insemination of eggs in sea water with a given amount and age of sperm suspension. This effect persists along with the increased survival of the sperm in these agents. The results demonstrate that the type of response given by the egg can be determined by the condition of the inseminating sperm. The spermatozoon evidently does not simply act in "all-or-none" manner, in the sense of operating a trigger mechanism in the egg.

6. Fertilization is also improved when the eggs are inseminated in the presence of Versene or glycine, but the effect is largely eliminated if the eggs are washed and inseminated in sea water. Swelling of the jelly-coat that occurs in these solutions is not an important factor in the results. Eggs that have been deprived of their jelly-coat behave similarly, showing improved fertilization in the solutions and no persistent pretreatment effect. Such eggs also show the improved response to treated sperm. It is concluded that if there is any effect of these agents on the egg in the direction of improving fertilizability such effect is readily reversed upon transfer to sea water and does not constitute a "maturating" effect.

7. Eggs of *L. pictus* and *S. purpuratus* develop normally to the pluteus stage in $10^{-3}$ molar Versene but not in $10^{-3}$ molar glycine.

## LITERATURE CITED

CHANG, M. C., 1947. Effects of testis hyaluronidase and seminal fluids on the fertilizing capacity of rabbit spermatozoa. *Proc. Soc. Exp. Biol. Med.*, **66**: 51–54.

CHANG, M. C., 1949. Effects of heterologous seminal plasma and sperm cells on fertilizing capacity of rabbit spermatozoa. *Proc. Soc. Exp. Biol. Med.*, **70**: 32–36.

Gray, J., 1928a. The effect of dilution on the activity of the spermatozoa. *Brit. J. Exp. Biol.*, **5**: 337–344.

Gray, J., 1928b. The senescence of spermatozoa. *Brit. J. Exp. Biol.*, **5**: 345–361.

Gray, J., 1931. The senescence of spermatozoa, II. *J. Exp. Biol.*, **8**: 202–210.

Greenberg, D. M., 1951. Metallic complexes of proteins and amino acids. Pp. 465–479 in: Amino acids and proteins. C. C. Thomas, Springfield, Illinois.

Hayashi, T., 1945. Dilution medium and survival of the spermatozoa of *Arbacia punctulata*. I. Effect of the medium on fertilizing power. *Biol. Bull.*, **89**: 162–179.

Lorenz, F. W., and A. Tyler, 1951. Extension of motile life span of spermatozoa of the domestic fowl by amino acids and proteins. *Proc. Soc. Exp. Biol. Med.*, **78**: 57–62.

Lyman, J., and R. H. Fleming, 1940. Composition of sea water. *J. Mar. Res.*, **3**: 134–146.

Martell, A. E., and M. Calvin, 1952. Chemistry of the metal chelate compounds. Prentice-Hall, Inc., New York.

Metz, C. B., 1945. The agglutination of starfish sperm by fertilizin. *Biol. Bull.*, **89**: 84–94.

Metz, C. B., and J. E. Donovan, 1950. Adjuvant action of amino acids and peptides in fertilizin agglutination of starfish sperm. *Science*, **112**: 755–756.

Rothschild, Lord, 1948. The physiology of sea-urchin spermatozoa. Senescence and the dilution effect. *J. Exp. Biol.*, **25**: 353–368.

Rothschild, Lord, 1951. Sea-urchin spermatozoa. *Biol. Rev.*, **26**: 1–27.

Rothschild, Lord, and P. H. Tuft, 1950. The physiology of sea-urchin spermatozoa. The dilution effect in relation to copper and zinc. *J. Exp. Biol.*, **27**: 59–72.

Runnström, J., 1947. Further studies on the formation of the fertilization membrane in the sea-urchin egg. *Arkiv Zool.*, **40A**, No. 1: 1–19.

Runnström, J., 1948. Membrane formation in different stages of cytoplasmic maturation of the sea-urchin egg. *Arkiv Zool.*, **40A**, No. 19: 1–6.

Runnström, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymology*, **9**: 241–327.

Runnström, J., and L. Monné, 1945. On changes in the properties of the surface layers in the sea-urchin egg due to varying external conditions. *Arkiv Zool.*, **36A**, No. 20: 1–23.

Runnström, J., L. Monné and L. Broman, 1943. On some properties of the surface layers in the sea-urchin egg and their changes upon activation. *Arkiv Zool.*, **35A**, No. 3: 1–32.

Runnström, J., L. Monné and E. Wicklund, 1946. Studies on the surface layers and the formation of the fertilization membrane in sea-urchin eggs. *J. Colloid Sci.*, **1**: 421–452.

Tyler, A., 1941. The role of fertilizin in the fertilization of eggs of the sea-urchin and other animals. *Biol. Bull.*, **81**: 190–204.

Tyler, A., 1948. Fertilization and immunity. *Physiol. Rev.*, **28**: 180–219.

Tyler, A., 1949. A simple, non-injurious, method for inducing repeated spawning of sea-urchins and sand dollars. *The Collecting Net*, **19**: 19–20.

✓ Tyler, A., 1950. Extension of the functional life span of spermatozoa by amino acids and peptides. *Biol. Bull.*, **99**: 324.

✓ Tyler, A., and E. Atkinson, 1950. Prolongation of the fertilizing capacity of sea-urchin spermatozoa by amino acids. *Science*, **112**: 783–785.

Tyler, A., and Lord Rothschild, 1951. Metabolism of sea-urchin spermatozoa and induced anaerobic motility in solutions of amino acids. *Proc. Soc. Exp. Biol. Med.*, **76**: 52–58.

Tyler, A., and T. Y. Tanabe, 1952. Motile life of bovine spermatozoa in glycine and yolk-citrate diluents at high and low temperatures. *Proc. Soc. Exp. Biol. Med.*, **81**: 367–371.

Wicklund, E., 1949. On the effect of albumin, ATP, trypsin, chymotrypsin, glucose and fructose on the activity of spermatozoa of sea-urchins. *Arkiv Zool.*, **42A**, No. 11: 1–10.

✓ Wicklund, E., and T. Gustafson, 1949. The effect of glycine on the membrane formation in the eggs of the sea-urchin *Strongylocentrotus droebachiensis*. *Arkiv Zool.*, **42A**, No. 12: 1–4.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

# SOME PHYSIOLOGICAL ASPECTS OF REPRODUCTION IN XIPHOPHORUS MACULATUS

HENRY H. VALLOWE[1]

*Whitman Laboratory of Experimental Zoology, University of Chicago, Chicago 37, Illinois*

Live-bearing fishes are being used extensively in biological research and instruction in genetics, embryology, endocrinology, pigmentation studies, and other fields to which these fishes can readily be adapted. Turner (1937) has carefully worked out the reproductive cycle of *Xiphophorus* from the viewpoint of ova development, period of fertilization, and superfetation. The present study is concerned with the reproductive cycle in this fish from the viewpoint of spermatozoa viability, selection and competition and the duration of ova production. Experiments have been conducted to show the number of young that can be born during the reproductive activity of the female and to show the duration of life of spermatozoa within the female genital tract and the competition and selection involved.

## Animals and Their Treatment

The fishes used in this experiment were kept in aquaria 20 × 25 × 20 cm., which had a layer of sand on the bottom about two inches deep. Each aquarium contained ample amounts of *Vallisneria* or *Sagitarria* planted along the back wall and two sides, allowing full observation from the front. Young fishes were kept in larger aquaria measuring 30 × 26 × 26 cm. until almost sexually mature; then they were segregated according to sex in the smaller type of aquarium. Prior to giving birth to young, gravid females were placed in aquaria of the smaller type heavily planted with hornwort (*Ceratophyllum*). After the young were born, the females were removed to other aquaria of the same size. The young were counted in the late afternoon of the day they were born and then returned to the same aquaria. No more than three sexually mature fish were kept in one aquarium.

The diet for mature fish throughout the experiment consisted of white worms, mosquito larvae, Daphnia, cyclops, prepared dry food and a dried and ground mixture of strained liver, spinach and bran flakes. Young fish were fed "micro worms" (Nematoda), prepared dry food, Daphnia and white worms. Mature fish were fed once a day. Young fish were fed three or four times daily.

Two kinds of water were used. More commonly the water was taken directly from the tap and allowed to stand in earthenware crocks or porcelain-lined tubs for about two weeks before being used. At other times water was used that had been filtered through charcoal. This was allowed to remain about a week in the tubs before being used. No adverse effect of the water used was noted during the course of the experiments. A routine schedule was maintained for cleaning the aquaria whereby each aquarium was thoroughly cleaned once each month.

---

[1] The writer wishes to thank Dr. W. C. Allee of the University of Florida for the help and kind supervision that he has given to this work. He also wishes to thank Dr. A. W. Bellamy of the University of California and Dr. Myron Gordon of the American Museum of Natural History for their generosity in supplying the fish that were used in these experiments.

The fish were kept in a greenhouse which has crude temperature control. In the winter months the temperature averaged about 75° F. during the day. During the last week of February, however, the steam supply was drastically reduced, and the temperatures were somewhat lower. During the summer months the control was much less perfect.

During the eight-months period that the fishes were kept under these conditions only three fishes died. Two of these, males of unknown age, slowly wasted away; the other, a female, died suddenly a few days before she should have given birth to her first brood of young. The reasons for these deaths could not be determined.

The species used in these experiments were *Xiphophorus maculatus, Xiphophorus variatus* and *Xiphophorus helleri—Xiphophorus maculatus* hybrids. Various color forms of *X. maculatus* were used. These included forms without any peduncular or caudal pattern, the one-spot pattern, the crescent pattern, the wag pattern, and the stippled, spotted, red, blue and red-bellied color forms. Matings were usually made between color forms and identity of young based upon color patterns.

## Experiments and Results

The experiments were of two types. In one experiment (Group I) female fish were isolated from contact with any male fish and records kept of the number of broods of young produced by them during the period of isolation. In some cases females were used which had been kept in aquaria for some time with males of their own species (Group I-A). Whether these females had given birth prior to being isolated cannot accurately be determined; therefore, the number of broods produced by these females is not considered as maximum. In another group (Group I-B) virgin females were used. After fertilization was accomplished these fish were isolated as in the previous experiment.

Another group of fish was kept in contact with males of their own species but of different color form and males of different species (Group II). In some cases the contact consisted only of the brief period of courtship and insemination (Group II-A). In other cases, (Group II-B), the contact with the male was constant except for the time that the female was removed for a few days prior to giving birth to a brood of young. (The term brood is used as defined by Turner (1937) to indicate a group of growing and differentiating ovocytes of approximately equal development up to the time of fertilization and also the embryos produced by the fertilization of these ova up to the time of birth.)

In some cases copulation was observed, but no young were produced by these females. These cases will be considered as Group III.

### *Group I-A*

A group of five females was kept isolated in small aquaria throughout the experiment. Records were kept of the number of broods, the number of young produced by these females and the interval of time between broods. These females had already been inseminated when the experiments began, and it is not known whether broods were produced by the females prior to this time. Therefore, the number of broods produced by these females cannot be considered as maximum. The results of this experiment are recorded in Table I. These results show a fairly consistent trend; that is, the number of young in the last brood is smaller than the usual number of young in the broods of each female. Fishes 0–1, 0–2, 0–4 and 0–5 were kept in contact with males after it was believed that they had produced their last broods.

At autopsy these fish showed the following results:

0–1. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male B-20, autopsy showed 16 large, deep amber ova; two large, light-colored ova; 16 embryos well developed; one embryo only slightly developed; and many small, white ova in the ovary.

0–2. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male M-10, autopsy showed 23 large, deep amber ova; two embryos about one-half developed; and many small, white ova in the ovary.

TABLE I

*Reproduction records of females of Group I-A*

| Females | Number in Brood A | Interval | Number in Brood B | Interval | Number in Brood C | Interval | Number in Brood D | Interval | Number in Brood E |
|---|---|---|---|---|---|---|---|---|---|
| 0–1 | 8 | 37 | 30 | 35 | 30 | 31 | 33 | 37 | 1 |
| 0–2 | 8 | 36 | 24 | 33 | 26 | 36 | 8 | 30 | 1 |
| 0–3* | 19 | 30 | 9 | 35 | 8 | 30 | 9 | | |
| 0–4 | 16 | 36 | 7 | 31 | 1 | | | | |
| 0–5 | 8 | 35 | 6 | | | | | | |
| 0–6 | 7 | 32 | 26 | 32 | 23 | 33 | 4 | | |

* Female 0–3 was kept in constant contact with male 0–20.

0–4. Ninety-eight days after delivery of the fourth brood and after 20 days of contact with male M-11, autopsy showed about 40 large, deep amber ova; several small, white ova; but no embryos.

0–5. One hundred and thirty-one days after delivery of the second brood and after 20 days of contact with male M-12, autopsy showed 34 embryos about three-quarters developed; several small, white ova; but no large, deep amber ova.

The mean interval between broods for these fish was 33½ days.

It seems justified to assume that these females ceased to produce young because of lack of viable spermatozoa within the ovary. With the possible exception of 0–4, none had reached the end of the reproductive period. Whether successful insemination was accomplished with female 0–4 is, of course, open to question. Copulation was observed, but it does not necessarily follow that the female was inseminated.

### *Group I-B*

In seven matings the females gave birth to a limited number of broods. They were kept isolated in small, heavily planted aquaria and examined every day for

TABLE II

*Reproduction records of females of Group I-B*

| Mating | Number of Broods | Total number of young | Time in days* | Days from last brood to autopsy |
|---|---|---|---|---|
| Female Male | | | | |
| C-1 × C-20 | 4 | 56 | 134 | 34 |
| C-5 × R-10 | 3 | 58 | 103 | 29 |
| C-7 × K-20 | 3 | 56 | 101 | 33 |
| K-2 × K-20 | 3 | 48 | 97 | 73 |
| K-3 × B-20 | 3 | 60 | 104 | 66 |
| K-6 × C-15 | 4 | 79 | 132 | 40 |
| N-3 × P-20 | 3 | 71 | 102 | 66 |

* From insemination to birth of last brood.

young. At the end of the experiment these females were autopsied and the condition of the ovary noted. These data are recorded in Table II. Evidently, the viable spermatozoa that were introduced at the insemination were depleted. Although the females were capable of producing more broods, none were produced because the lack of viable spermatozoa in the ovary or genital tract made fertilization impossible.. Popular literature about aquarium fishes sets the upper limit for the number of broods produced in isolation at eight broods. This limit was not reached in these experiments. In all instances autopsy revealed that the ovary contained large, deep amber ova; small, white ova; but no embryos.

TABLE III

*Reproduction records of females of Group II-A*

| Female | Insemination date | Interval to Brood A | Interval to Brood B | Interval to Brood C | Interval to Brood D | Interval to Brood E |
|---|---|---|---|---|---|---|
| C-1 | II-6 | 38 | 32 | 32 | 33 | |
| C-2 | VI-6 | 48 | | | | |
| C-3 | VI-7 | 45 | | | | |
| C-4 | VI-12 | 42 | | | | |
| C-5 | III-15 | 41 | 31 | 31 | | |
| C-6 | V-15 | 42 | 29 | | | |
| C-7 | III-13 | 40 | 31 | 30 | | |
| C-8 | IV-26 | 50 | 31 | | | |
| Ca-2 | II-11 | 40 | 32 | 30 | 32 | 28 |
| Ck-1 | VI-6 | 37 | | | | |
| K-1 | II-6 | 57 | 31 | 31 | 27 | |
| K-2 | II-4 | 39 | 26 | 32 | | |
| K-3 | II-4 | 42 | 32 | 30 | | |
| K-5 | V-31 | 44 | | | | |
| K-6 | II-4 | 40 | 32 | 28 | 32 | |
| N-2 | IV-26 | 42 | 30 | | | |
| N-3 | II-6 | 39 | 31 | 33 | | |
| P-3 | VI-5 | 31 | | | | |
| P-5 | V-18 | 35 | | | | |

### *Group II-A*

Soon after the experiments were begun it was noted that the virgin females usually did not give birth to their first brood until about 40 days after fertilization. Since copulation had been observed in each case and the female isolated from all contacts immediately after insemination, accurate dating of the period between insemination and birth of the brood was possible. Table III shows the periods in days between insemination and birth of first brood.

This table shows that for the cases listed the average period between insemination and birth of first brood was 41.7 days. When this period is compared with the periods between subsequent broods for these females, it is evident that some factor is involved which delays the birth of the first brood. What this factor is was not ascertained in this study.

That it is due to some delayed action on the part of spermatozoa is not plausible in the light of one experiment in which a female was inseminated by two males at different times. Forty-two days after insemination by the first male the female gave birth to her first brood of young. On that day the female was fertilized by the second male. Thirty days after this second insemination the second brood was born. Of the 41 young, 28 were of the same phenotype as the second male. In this case the spermatozoa fertilized ova that developed in the normal thirty-day period.

### *Group II-B*

Several attempts were made to establish the degree of competition between the spermatozoa of different males. In some cases females were used which had been inseminated by males of their own species or hybrid type. After giving birth to broods of young, these females were placed in constant contact with males of different species or males of the same species but of a phenotype different from the original male. The results of these experiments are as follows:

1. A *X. maculatus* female, K-1, was inseminated by a male *X. variatus,* V-20. Four days after the birth of the second brood from this mating (a period of 92 days from insemination) the female was placed in constant contact with a male *X. maculatus*. During the contact with this male, two broods of young were born. Although the males differed phenotypically and the young produced from ova fertilized by spermatozoa of the *X. maculatus* male could have readily been distinguished from young produced from ova fertilized by the *X. variatus* male, no young could be assigned to the *X. maculatus* male. Copulation was observed after the second male was introduced. Whether insemination took place cannot be ascertained.
2. A *X. maculatus* female, N-2, was fertilized by a *X. maculatus* male, B-20. On the day the first brood was born contact was made with a *X maculatus,* male, 0–21. In the second brood 28 of the 41 young were of the same phenotype as male 0–21 and distinct from the phenotype of male B-20.
3. A hybrid *X. helleri–X. maculatus* female was kept in constant contact with a male *X. maculatus,* K-20. No other fish were kept with this pair. During 136 days of contact, the female gave birth to four broods of 56 young. Two of the fourth brood of fifteen showed the phenotype of male K-20. A fifth brood of nine contained no young of the same phenotype as male K-20.

4. A similar experiment was set up with a brood sister of the female described in (3) above. This female had previously given girth to two broods of young before being placed with male 0–22. During 107 days of constant contact with male 0–22 the female hybrid gave birth to four broods of 52 young. Three young of the fourth brood showed the phenotype of male 0–22.

5. A third hybrid female was placed in contact with a male *X. maculatus,* W-1, after having been inseminated by a male hybrid from the same brood. During 116 days of contact, the female had given birth to four broods. None showed the phenotype of male W-1. The male died before the fifth brood of seven young was born. None of this brood had the phenotype of male W-1.

These experiments, although incomplete and lacking reciprocal crosses, show that once a female has been fertilized by a male of either the same species or a different species, the spermatozoa of the second male can fertilize ova while the spermatozoa of the first male are still viable and fertilizing ova which are in the same broods. These experiments also give an indication of the degree of competition that exists among viable spermatozoa within the ovaries of female viviparous fishes.

A pair of *X. maculatus* was kept in a small aquarium isolated from other fishes to serve as controls and to establish what degree of relationship, if any, existed between constant insemination and the number of young and interval between broods. These results are listed in Table I for female 0–3. At autopsy 42 days after the birth of the last brood this female contained 33 large ova of a very pale, almost transparent color. Usually ova at autopsy are deep amber in color. After development begins the deep amber ova remain the same color and do not change until the embryos are evacuated from the female's body. If any of the yolk material remains at birth, it soon changes to an opaque yellow. No autopsy revealed embryos developing from ova of the pale, transparent type.

### *Group III*

The normal procedure for mating fish in all these experiments was to place the female in a small aquarium which had been occupied for at least a day by an isolated male. In most cases the courtship began almost immediately or as soon as the male was aware of the female in the aquarium. Copulation usually took place after twenty minutes or less of courtship. One lasting contact was considered as sufficient, and an effort was made to limit the mating to only one contact. In some cases females never produced young, although copulation with a lasting contact was observed. Females C-8, N-1, N-4, P-2 and P-4 had contact with only one male but never produced young from these matings. Female C-8 was later mated with another male, C-23, and produced broods from this mating. Females Ca-1, Ck-1, K-4 and P-1 had contact with two males in rapid succession. This was an attempt to have spermatozoa from two different males introduced into the genital tract of the female at as nearly the same time as possible. In two cases, females Ck-1 and P-1, copulation with the second male was accomplished within two minutes after copulation with the first male. Female Ck-1 was later mated with another male, L-20, and produced broods of young.

Whether this failure to produce broods of young can be attributed to lack of insemination and subsequent fertilization is open to question because other factors such as immaturity may have been responsible. It is noteworthy to mention here that all four attempts to inseminate females with the spermatozoa of two males met with failure. In the total of nine cases of failure in insemination seven males were used (males R-10, B-20, O-21, V-10, C-23, S-10 and X-15). In other matings males R-10, B-20, O-21, V-10, C-23 and X-15 were shown to have produced viable spermatozoa. Male S-10 died before being mated again. Autopsy of female Ca-1 showed that the ovary contained 41 large, deep amber ova. The remainder of this group of females is being used in other experiments and cannot be autopsied at this time. Attempts at hybrid matings of female *X. maculatus* and male *X. helleri* met with failure; no copulation was observed.

## Discussion

An attempt has been made, by means of controlled mating, to determine what some of the factors are that affect reproduction in *X. maculatus*. It has been shown by Turner (1937, 1940), Hopper (1943), Wolf (1931) and others that a definite ovarian cycle exists in the female of this species. The cycle may be briefly outlined as follows:

1. Upon completion of development within the follicles of the ovary, embryos are evacuated to the ovarian cavity from which they descend the short genital tract (oviduct) to the exterior of the female's body.
2. As this brood is developing within the ovary, a group of ova which is approximately equal in number to the embryos is becoming larger, accumulating yolk material and approaching a state in which fertilization is possible.
3. A few days after the brood is born the ova are fertilized in the ovarian follicles by the spermatozoa within the ovary. Winge (1922) shows a photomicrograph of spermatozoa lying ready in the ovary of a *Lebistes* female. The heads of the spermatozoa are as near the immature egg as possible.
4. A third group of ova begins to enlarge and the cycle is continued. Bellamy (1924) states that female *X. maculatus* are capable of producing as many as ten broods.

To my knowledge no female *X. maculatus* that has not been inseminated by a male of the same or closely allied species has given birth to young. Hubbs and Hubbs (1946) report an interesting case of *Mollienisia formosa* in which no males of the species are known except from experiments of masculinizing females with gonadotrophic hormones. The female of the species has never been known to reproduce parthenogenically but must first mate with a male of another species before giving birth to young. The young show no signs of paternal inheritance. Hubbs and Hubbs regard this as a species permanently fixed diploid requiring only the stimulus of spermatozoa to initiate development.

That a competition and selection exist among the spermatozoa within the ovary and genital tract of the female is shown in the experimental data for Group II-B. In these experiments various hybrid matings were attempted. These are as follows: (1) a female *X. maculatus* with a male *X. variatus* and male *X. maculatus*, (2) a female *X. maculatus* with two male *X. maculatus* of different phenotypes, (3) three female *X. helleri–X. maculatus* hybrids with three *X. maculatus* males of different phenotypes.

In the first mating the *X. maculatus* male never successfully fertilized any ova of two broods totaling 59 in number, while the male *X. variatus* fertilized ova producing 117 young. The *X. variatus* male was in contact with the female only long enough to effect one insemination; the *X. maculatus* male was placed with the female 92 days after insemination by the *X. variatus* male and four days after the birth of the second brood. This male remained in constant contact with the female for 55 days until the birth of the fourth brood. If the *X. maculatus* spermatozoa had fertilized any of the 59 ova of the third and fourth broods, the young would have shown the crescent tail pattern which was carried by the male. Gordon (1947) has shown that a series of seven dominant autosomal allelic genes controls the pattern of peduncular and caudal pigmentation in *X. maculatus*. Throughout these experiments these data have been used to establish identity and paternal relationship.

All the surviving fish of the first brood produced from this *X. maculatus–X. variatus* cross have differentiated as males. There are differences in the sex-determining mechanisms of the two species. In *X. variatus* the female is the homogametic sex; in domestic *X. maculatus* the male is the homogametic sex. Gordon (1944) confirms the findings of Bellamy (1936) and Kosswig (1935) that all the hybrids from matings of domestic male *X. maculatus* and female *X. variatus* are males. The hybrid mating described in these experiments uses the two heterogametic sexes. The fact that only ten of the twenty young have survived to sexual maturity suggests that other factors may be involved. Perhaps chance has produced such a sex ratio. None of the young from the subsequent broods have reached sexual maturity, and the small number of young in the first brood does not justify any conclusion.

In the second mating described the first male was in contact with the female for a period of 26 days. Forty-two days after the female was introduced into the aquarium containing the male the first brood was born. Beginning on this day the female was placed in contact with the second male for a period of 29 days. On the following day the second brood of 41 was born. Twenty-eight of this brood had the peduncular pattern of the second male; thirteen resembled the first male. The spermatozoa of the second male succeeded in fertilizing the majority of the ova of the second brood. Reference to the other matings in which only one male was used leads to the thought that without intervention of the spermatozoa of the second male, the spermatozoa of the first male would have fertilized the majority of all the ova of the second brood. This indicates that a competition exists among spermatozoa within the ovary or genital tract of the female. The subsequent selection, which is a product of this competition, might have its foundations in the different ages or different quantities of spermatozoa, differential placement within the genital tract, or more subtle differences in viability, size or other factors.

In those experiments in which hybrid females were used, an attempt was made to ascertain the degree of competition between spermatozoa of different species. These females were of a strain that has been bred in commercial hatcheries to include the *X. maculatus* gene for red body color. Gordon (1946) has traced the development of a similar strain which has been developed to include the *X. maculatus* gene for the comet tail pattern in the cytoplasm of the swordtail, *X. helleri*. The red swordtail-platyfish hybrids have been bred by back crosses to the wild-type swordtail to produce a fish that is identical in body form and size, behavior and taxonomic characters of dorsal fin ray count and lateral line scale count with the wild-type swordtail. In these experiments the females used had previously been inseminated by a male of their own type (in reality a brood brother). They were then placed with *X. maculatus* males. In two of these matings hybrid young carrying the color patterns of the *X. maculatus* males were produced. The third mating was unsuccessful in producing any hybrid young. In one case two hybrids were produced after 136 days of contact; in the other three hybrids were produced after 137 days of constant contact. These periods of contact before hybrid young were produced compared favorably with the periods of 97 to 145 days for the production of young by females in isolation (Groups I-A and I-B). In light of these data there is an indication that the spermatozoa of the *X. maculatus* male are selected against, and that they can fertilize ova only when the spermatozoa of the *X. helleri* male are partially depleted or reduced in number below a critical point.

Another factor which should be considered here is the difference between the courtship and mating behavior of these two species. Clark *et al.* (1948) after a study of their behavior concluded that differences between these fishes do exist and that such psychological barriers can effectively prevent their hybridization in natural situations.

These experiments on the effect of spermatozoa viability, competition and selection have certain general implications as they stand, regardless of the fact that the analyses of the underlying mechanisms are incomplete or entirely wanting. These may be summarized briefly as: (1) offering suggestions concerning the regulation of the reproduction of *X. maculatus* in natural habitats; (2) pointing out a barrier that exists in nature between two sympatric species of livebearing Cyprinodontes; (3) supporting the concept that there are simple social factors such as competition and selection, in operation below the level usually regarded as social, and that these factors can operate in small and subtle ways.

## SUMMARY

1. Experiments have been conducted which show that *Xiphophorus maculatus* females which have been successfully inseminated can continue to give birth to young after isolation from contact with male fishes. Although no maximum limit has been established, three to five broods of young has been shown to be the general trend.

2. Females ceased to produce young because of the lack of viable spermatozoa within the ovary. After the spermatozoa within the ovary cease to fertilize ova, the female can be inseminated again and can again produce broods of embryos.

3. By hybrid matings support has been given to the concept of isolating factors between *X. helleri* and *X. maculatus* in nature. Where attempts have been made to inseminate female *X. helleri* with *X. maculatus* spermatozoa, the results were very poor or failed completely.

4. The period of time between insemination and birth of the first brood is longer than the periods of time between subsequent broods produced from the same insemination.

5. Copulation by a female with two male fish in rapid succession failed to produce broods of embryos in these experiments.

6. The general implications of these experiments are suggested.

## LITERATURE CITED

Bellamy, A. W., 1924. Bionomic studies on certain teleosts (Poeciliinae). I. Statement of problems, description of material, and general notes on life histories and breeding behavior under laboratory conditions. *Genetics,* **9**: 513–529.

Bellamy, A. W., 1936. Interspecific hybrids in Platypoecilus: one species ZZ-ZW; the other XY-XX. *Proc. Nat. Acad. Sci.,* **22**: 531–535.

Clark, E., L. R. Aronson and M. Gordon, 1948. An analysis of the sexual behavior of two sympatric species of poeciliid fishes and their laboratory induced hybrids. *Anat. Rec.,* **101**: 692–693.

Gordon, Myron, 1944. Similar sex-determining mechanisms in wild populations of *Platypoecilus xiphidium, variatus* and *maculatus. Rec. Gen. Soc. Amer.,* **13**: 19.

Gordon, Myron, 1946. Introgressive hybridization in domestic fishes. *Zoologica,* **31**: 77–88.

Gordon, Myron, 1947. Speciation in fishes. *Advances in Genetics,* **1**: 95–132.

Hopper, Arthur F., 1943. The early embryology of *Platypoecilus maculatus. Copeia,* **4**: 218–224.

Hubbs, Carl, and Laura C. Hubbs, 1946. Breeding experiments with the invariably female, strictly matroclinous fish, *Mollienisia formosa. Genetics,* **31**: 218.

Kosswig, Curt, 1935. Die Kreuzung zweier XX-bzw. XY-geschlechter miteinander und der ersatz eines Y-Chromosoms einer Art durch das X-Chromosom einer anderen. *Der Züchter,* **7**: 40–48

Turner, C. L., 1937. Reproductive cycles and superfetation in poeciliid fishes. *Biol. Bull.,* **72**: 145–164.

Turner, C. L., 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. *J. Morph.,* **67**: 59–90.

Winge, O., 1922. A peculiar mode of inheritance and its cytological explanation. *J. Gen.,* **22**: 137–144.

Wolf, L. E., 1931. The history of the germ cells in the viviparous teleost *Platypoecilus maculatus. J. Morph. and Physiol.,* **52**: 115–153.

# TEMPORAL VARIATIONS IN HISTOLOGICAL APPEARANCE OF THYROID AND PITUITARY OF SALAMANDERS TREATED WITH THYROID INHIBITORS[1]

AMBROSE J. WHEELER[2]

*Department of Biology, The Catholic University of America, Washington, D. C.*

The fact that thiourea and a number of related chemical compounds are capable of interfering in some way with the normal activity of the vertebrate thyroid gland is now well known. Administration of these substances to common laboratory mammals causes an almost immediate decrease in the thyroid hormone level as evidenced by a lowered rate of metabolism and other symptoms of hypothyroidism. With continued treatment the thyroid gland becomes hypertrophic and hyperplastic, presumably because the decreased content of thyroid hormone in the blood induces a compensatory over-production of thyrotrophic hormone by the pituitary gland. The thyroids of treated animals may thus become markedly enlarged and these thyroid-inhibiting drugs are therefore often referred to as goitrogens.

An extensive literature is available concerning the effects of various goitrogens upon the morphology and physiology of the thyroid gland and the possible mechanism of action of the drugs in laboratory mammals and in the human subject. (See reviews of Charipper and Gordon, 1947; Trotter, 1949; Astwood, 1949; Pitt-Rivers, 1950; Lever, 1951.) Relatively little work has been done on the effects of these compounds on lower vertebrates. However, there is sufficient information available to indicate that they are effective to greater or lesser degree in all types of vertebrates and probably act in essentially the same way in all (Lynn and Wachowski, 1951). Among amphibians the effectiveness of thiourea and related compounds is readily shown by their ability to inhibit metamorphosis in the tadpole (Gordon, Goldsmith and Charipper, 1943; Hughes and Astwood, 1944; Lynn and De Marie, 1946; Koch, 1948; Harms, 1949; Delsol, 1948). However, study of the thyroid glands of treated tadpoles has revealed that although the histological changes observed are similar to those reported for mammals, they are less marked, and after long-continued treatment a decrease in thyroid size and activity may be observed (Gordon, Goldsmith and Charipper, 1945). This decrease also occurs in the adult frog, and Joel, D'Angelo and Charipper (1949) found that after such "regression" the gland may be reactivated by administration of thyrotrophic hormone. It thus appears that the indications of decreased activity observed in the thyroid after long-continued treatment with goitrogens are due to the exhaustion or the impairment of the pituitary thyrotrophic function. Adams (1946) reported that adult salamanders (*Triturus viridescens*) exhibit relatively slight thyroid hyper-

---

[1] This paper is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Catholic University of America.

I am indebted to Dr. W. Gardner Lynn for suggesting this problem and for many helpful suggestions during the course of the work.

[2] Present address: University of Portland, Portland, Oregon.

plasia after long-continued treatment with thiourea. It has been suggested (Lynn and Wachowski, 1951) that this may also be due to a "regression" similar to that noted in the frog.

The present investigation is a study of the histology of both the thyroid and pituitary glands in salamanders treated for periods varying from 5 to 90 days with several different concentrations of four different goitrogens. This study was undertaken in an attempt to ascertain more precisely the effects of these various treatments upon the thyroid, and to find out whether there are any histological changes in the pituitary which can be correlated with the thyroid response.

## Materials and Methods

The salamanders used in the present experiments were adult specimens of *Desmognathus fuscus brimleyorum* Stejneger obtained from Dr. Charles Burt of Topeka, Kansas. The animals were kept under laboratory conditions for about a month before use. During this time they exhibited normal activity and a very low mortality rate.

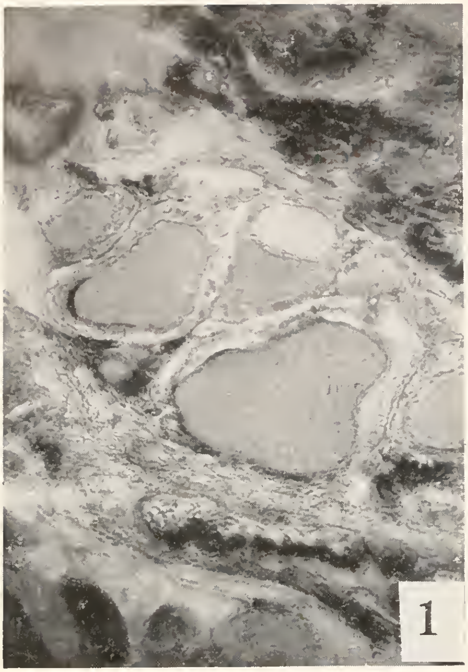
Treatment with thyroid-inhibitors was carried out simply by maintaining the specimens in culture solutions consisting of the appropriate concentrations of the drugs in tap-water. The animals were kept in large finger bowls containing one liter of solution, with seven or eight animals to a bowl. The solutions were changed weekly. The salamanders were fed earth-worms about once a month. At regular intervals, control and experimental animals were removed from the solutions and anesthetized with MS 222. From these animals thyroids and pituitaries were removed. The thyroid glands were fixed in Bouin's fluid and the pituitaries in Zenker's fluid. After two hours fixation, which proved to be sufficient, the material was washed in running water, dehydrated in Cellusolve, cleared in oil of wintergreen and embedded in paraffin. Thyroids were sectioned at 8 $\mu$ and stained with Mallory's triple stain. Pituitaries were sectioned at 5 $\mu$ and stained according to the technique of Pearse (1950).

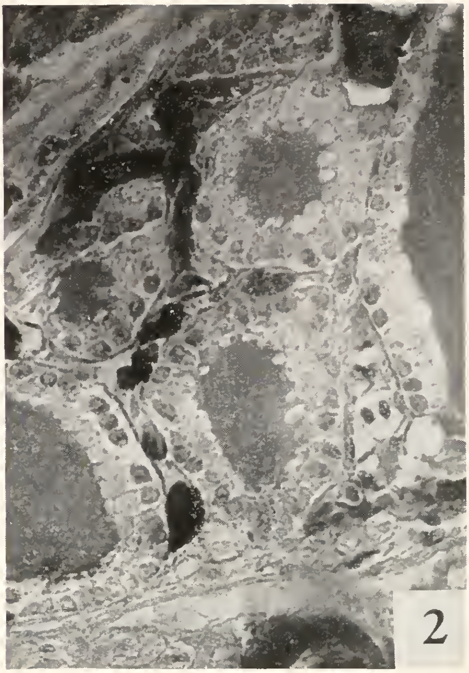
In addition to several sets of controls kept in tap-water, the following concentrations of goitrogens were used: 0.005%, 0.01%, 0.05%, 0.1%, 0.3%, 0.5% and 1% thiourea; 0.005%, and 0.01% phenylthiourea; 0.05% allylthiourea; 0.05% thiouracil.

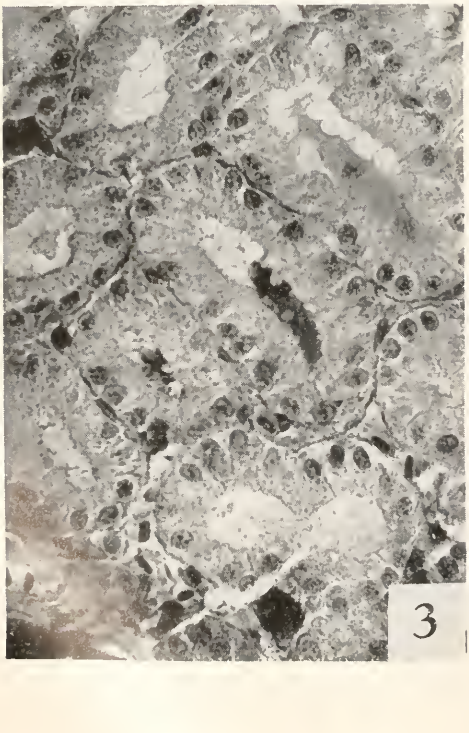
## Results

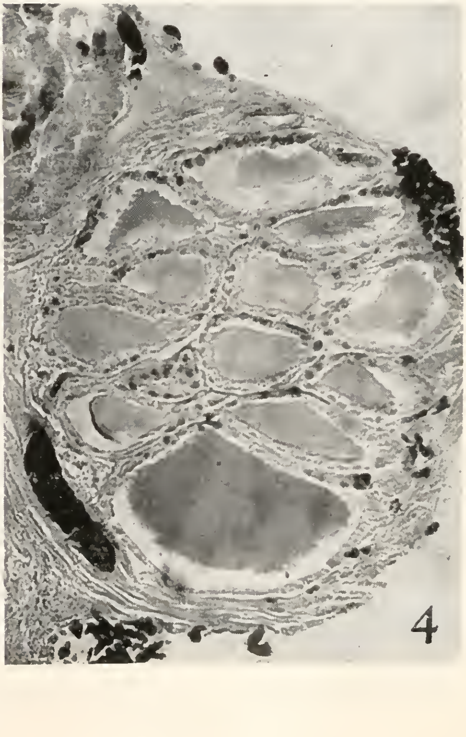
### *A. Effect of treatment with goitrogenic agents upon the histology of the thyroid gland*

All of the treatments used in the present study were effective in producing well-defined histological changes in the thyroid gland of the salamander. However, as one would expect, the effects observed and the time required for their appearance varied with the different drugs and with different concentrations of the same drug. These effects are similar to those recorded in the literature for other vertebrates such as the rabbit (Baumann, Metzger and Marine, 1944) and the chick (Mixner, Reineke and Turner, 1944) and are in particularly close agreement with those reported for other amphibia (Gordon, Goldsmith and Charipper, 1943; Adams, 1946; Gasche and Druey, 1946; Blakstad, 1949; Lynn, 1948; Joel, D'Angelo and Charipper, 1949).


1


2


3


4

The general sequence of changes observed in the thyroid following treatment with a goitrogen may be briefly outlined as follows. The first indication of an effect is usually seen in an increased vascularity of the gland. This is often grossly discernible in the intact thyroid and is readily detectable in sectioned material where the hyperemia is indicated by enlarged vessels and an increased number of blood cells (Fig. 2). This change is usually followed in a short time by the appearance of chromophobe droplets ("vacuoles") within the epithelial cells and around the periphery of the colloid mass (Fig. 2). These intracellular and intrafollicular vacuoles are generally considered as reliable indications of increased activity in the gland and they continue to increase in size and number with time. As the vacuolization progresses, the form of the follicular epithelium gradually changes from flat or cuboidal to high columnar and at the same time the staining reaction of the intrafollicular colloid undergoes an alteration from acidophilic to basophilic. Following this the amount of intrafollicular colloid may be greatly decreased so that many follicles have their lumina completely obliterated (Fig. 3), the walls of the follicles being extremely thick and often much folded. Hyperplasia may occur, as indicated by an increase in the number of mitotic figures observed in the epithelium and the whole gland may become grossly hypertrophied. However, such hypertrophy was never so marked as that which is produced by comparable treatment in mammals or even in larval amphibians. After long-continued treatment with relatively high concentrations of goitrogens the appearance of the tissue may return almost to normal with decrease in the height of the epithelium and renewed storage of acidophilic intrafollicular colloid (Fig. 4). The basis for this "regression" will be discussed at length later.

It will be remembered that in these experiments a rather wide range of concentrations of thiourea was investigated while only a few concentrations of three other goitrogens were employed. Using as a basis the foregoing general account of the effects of these substances upon the thyroid, the detailed results of each of the experimental treatments will now be considered.

1. 0.005% thiourea

Thyroids of animals raised in this concentration of thiourea showed no differences from control glands (Fig. 1), until 15 days after the beginning of treatment. At this time there was a definite increase in vascularity and a few intracellular and intrafollicular vacuoles were apparent in some but not all follicles of the experimental animals. By the 25th day of treatment the staining reaction of the colloid mass was basophilic but the follicular epithelium was still cuboidal and the number of vacuoles had not significantly increased. Specimens killed at later periods up to the 88th day of treatment, when the experiment was terminated, exhibited essentially this same condition except for a slight increase in the number of intrafollicular

---

FIGURE 1. Photomicrograph of normal thyroid gland in *Desmognathus fuscus brimleyorum.* × 150.

FIGURE 2. Photomicrograph of a thyroid gland from an animal treated for 25 days with 0.05% thiouracil. × 280.

FIGURE 3. Photomicrograph of a thyroid gland from an animal treated for 25 days with 0.05% thiourea. × 280.

FIGURE 4. Photomicrograph of a thyroid gland from an animal treated for 85 days with 0.05% thiouracil. × 150.

vacuoles. In none of the specimens studied did the epithelium become columnar nor was there any indication of a decrease in colloid volume.

2. 0.01% thiourea

Clear-cut effects appeared in this series within 10 days after the beginning of treatment. At this time there was marked hyperemia, the follicular epithelium had become low-columnar, both intrafollicular and intracellular vacuoles were abundant and in most follicles the colloid mass, while still acidophilic at the center, showed a wide peripheral zone of basophilic material. These features persisted in the later specimens but the amount of colloid in the gland gradually decreased so that by the 37th day of treatment many follicles appeared to be completely empty. By 72 days the epithelium had become extremely high and the colloid content of the gland was even further diminished. This condition still existed on the 90th day when the experiment ended (Fig. 3).

3. 0.05% thiourea

The effects of this treatment corresponded with those obtained with 0.01% thiourea during the early phases of the experiment. At 10 days the epithelial height was only moderately increased but vacuolization, increased vascularity and basophilia in the colloid were clearly evident. The epithelium became tall columnar by the 15th day, however, and by the 25th day the amount of stored colloid in the gland had been markedly reduced (Fig. 3). This represents the maximum effect obtained with this concentration and the histological condition presented by the thyroid remained unchanged throughout the remainder of the experimental period (72 days).

4. 0.1% thiourea

Specimens exposed to this concentration for 10 days showed striking effects which included not only hyperemia and increased vacuolization but also hypertrophy of the epithelium and decrease in colloid volume. The latter had already proceeded to such a degree that many follicles were completely collapsed. This condition persisted through the 54th day of treatment but in the thyroids of animals killed at later periods there were some indications of a decrease in activity of the thyroid. These indications consisted in some slight reduction in epithelial height and a gradual but rather marked increase in the stored colloid volume. In the specimens tested longest (85 days) some acidophilic colloid was present.

5. 0.3% thiourea

As in the immediately preceding series the earliest specimen studied (10 days) already showed what might be considered almost maximal effects. Material treated for longer periods also corresponded with that seen in the specimens treated with 0.1% thiourea so that this series shows no significant difference in the effectiveness of these two concentrations.

6. 0.5% thiourea

Although animals treated with 0.1% and 0.3% showed marked histological changes in the thyroid within 10 days, specimens treated with 0.5% thiourea did not show such effects. Even up to 25 days of treatment the thyroids of these animals differed from those of the controls only in displaying a slight hyperemia, low columnar epithelium and a moderate degree of intrafollicular vacuolization. By the 37th day the vacuolization had greatly increased and some follicles had a depleted colloid content. However, this seems to have been the period of maximum effect for in specimens examined after the 37th day the colloid content was gradually increased, the epithelium decreased in height and by the 72nd day of treatment the gland had almost regained the appearance seen in the controls.

7. 1.0% thiourea

The effects of this, the highest concentration of thiourea used, were similar to those obtained with 0.5% thiourea. No significant changes occurred until the 25th day. These increased to a maximum at about the 54th day and then there was a well-defined "regression." However, the decrease in activity had not proceeded so far as to produce a gland of normal appearance when the experiment was terminated at 90 days.

8. 0.005% phenylthiourea

This concentration of phenylthiourea proved more effective than the same concentration of thiourea. Animals treated for 10 days exhibited numerous intracellular and intrafollicular vacuoles and marked hyperemia of the gland. By the 15th day the epithelium had become extremely high and depletion of the colloid content was already far advanced. This same condition was presented by the glands of animals treated up to 54 days but following this a well-defined decrease in activity occurred which was more striking than that seen in any of the thiourea treatments. The height of the epithelium gradually decreased until by 72 days all follicles were lined by flattened cells. Intracellular and intrafollicular vacuoles entirely disappeared and the colloid became dense, homogeneous and completely acidophilic. The gland of the experimental animal at this time thus gave the appearance of a much more inactive thyroid than is seen in the average control.

9. 0.01% phenylthiourea

The effects with this concentration were even more striking than those obtained with 0.005% phenylthiourea. After only 10 days treatment much of the stored colloid had disappeared and by the 25th day there was almost no colloid present in any of the follicles. Specimens given longer treatment show evidence of "regression" although this is not so marked as in the immediately preceding series.

10. 0.05% phenylthiourea

This concentration represents the maximum solubility for this drug and it proved toxic to these animals in a short period. The thyroid of one specimen which survived for 5 days in this concentration was available for study. It is of interest since it already showed increased epithelium height and extreme vacuolization and thus furnished some indication that this concentration is probably even more effective than the preceding.

11. 0.05% thiouracil

This treatment gave no significant results until the 25th day. At this time the epithelium height had increased, intrafollicular vacuoles were abundant and the gland had become hyperemic (Fig. 2). The effects became more pronounced at later stages but by the 85th day there was clear evidence of decreased activity, the epithelium having become low columnar to cuboidal and the colloid having increased in amount (Fig. 4).

12. 0.05% allylthiourea

The effect of this treatment was not apparent until the 37th day but by the 54th day quite marked effects were obtained, the colloid being completely exhausted and the whole gland hypertropic. As in the case of treatment with 0.05% thiouracil, this was later followed by decreased activity, the epithelium becoming low and the colloid abundant in the later stages studied.

### *B. Effects of treatment with goitrogenic agents upon the histology of the pituitary*

The fact that the thyroid gland is activated by a thyrotrophic principle had well been demonstrated in a number of ways. But precisely which cells of the pituitary are responsible for the elaboration of this hormone is still open to question. One of the aims of the present study was to obtain some evidence on this point.

The generally accepted concept of the mode of action of the goitrogenic substances used in this work is as follows. These drugs interfere in some way with the normal synthesis of the thyroid hormone. As a result of this the thyroid hormone level in the blood is decreased and this decrease stimulates the anterior lobe of the pituitary to produce and/or release its thyroid-activating hormone. The histological changes in the thyroid thus result from stimulation by the thyrotrophic hormone. If the goitrogenic substances are administered continuously, the thyroid, despite its increased cell height and general hypertrophy, continues to be hypofunctional and therefore the pituitary continues to release the thyrotrophic hormone and a goiterous condition results. If a particular cell type in the pituitary is responsible for secretion of the thyrotrophic hormone it would be expected that in animals in which these cells have been over-stimulated by long-continued treatment with goitrogens, some histological changes involving them should be observable. In the material under investigation there is also the possibility that in those animals in which later decreased activity of the thyroid was observed, some histological evidence of the basis for this decrease might be seen in the pituitary. It has been suggested (Joel, D'Angelo and Charipper, 1949) that the thyroid "regression" comes about because the thyrotrophic hormone-secreting cells of the pituitary, after long-continued over-activity, become exhausted and fail to secrete any longer. This would result in a reduction in the thyroid-activating principle in the blood and a consequent return of the thyroid to a normal, or more probably, to an extremely inactive condition.

Although both the pituitary and thyroid glands of all animals were studied, detailed consideration of the histology of the pituitary will be limited to points which bear upon the question of secretory specificity of the cells of the anterior lobe. It is obvious that the most important material to be considered will be pituitaries from animals whose thyroids showed maximum hypertrophic effects and pituitaries from animals which showed the most clear-cut evidence of decreased thyroid activity after long-continued treatment. These, along with pituitaries of control animals, form the basis for the account which follows.

The general appearance of the anterior lobe of the pituitary in the salamander, *Desmognathus fuscus brimleyorum,* with the staining technique employed here is as follows. The acidophilic elements show a clear yellow-staining cytoplasm with a relatively large centrally located nucleus; the basophilic elements exhibit a somewhat excentric nucleus and a cytoplasm which is heavily granulated, and magenta-staining; the chromophobic elements, which are difficult to identify in many cases, show a pale cytoplasmic background and a heavily staining nucleus. The nucleus stains dark blue in all three types of cells.

The first indication of changes in the pituitary were found in animals whose thyroids already exhibited well-marked signs of inhibition. Study of a series of animals in which the thyroids showed progressive activation revealed that the pituitary glands were undergoing concurrent changes characterized by a gradual increase in the proportion of basophilic elements and a decrease in the relative numbers of acidophilic elements. At the same time the chromophobic elements exhibited an increased granulation which may possibly represent transition toward a basophil type.

A study of the pituitaries of specimens whose thyroids exhibited decreased activity after long-continued treatment revealed that the basophilic elements were still predominant but there was evidence of some degenerative change in these cells. The cell outlines tended to be indistinct, the cytoplasm stained less uniformly and many cells had distorted, seemingly pycnotic, nuclei. There was no evidence in this material of any increase in the relative numbers of acidophilic elements. These results tend to support the concept that the decreased activity of the thyroid after long-continued treatment with goitrogens is not due to any return of the pituitary to normality but rather to an abnormality of the basophil cells of the anterior lobe probably resulting from their over-activity and consequent exhaustion.

## Discussion

As has been noted, all of the treatments used in the present study produced well-defined histological changes in the thyroid gland but the extent of the changes and the time required for their appearance differed with the treatment. Since thiourea was the only drug for which a wide range of concentrations was tested, conclusions concerning the relation between concentration and goitrogenic effects must be limited to this substance. It will be remembered that treatment with 0.005% thiourea, the lowest concentration tested, was not sufficient to cause maximal activation of the thyroid during the experimental period of approximately three months. With 0.01% thiourea detectable changes in the thyroid appeared sooner and maximal effects were apparent by the 72nd day of treatment. The next higher concentration, 0.05% thiourea, caused an even earlier maximal activation. It appears, however, that the optimal concentration with respect to rapidity of effect lies in the region of 0.1% to 0.3%. These concentrations gave essentially the same results. The thyroids of animals treated for only 10 days appeared to be already fully activated and the condition remained unchanged during the subsequent two months of treatment. During the third month of the experiment the thyroids of animals given these concentrations exhibited signs of a gradual decrease in activity which resulted finally in a histological condition indicating marked inactivity of the gland. The two highest concentrations of thiourea tested, 0.5% and 1.0%, caused less marked changes in the thyroid than did 0.3% or 0.1%. It thus appears that in the present material, the goitrogenic effects of thiourea administered in the culture medium increase with the concentration of the drug up to an optimal concentration at 0.1% to 0.3% and decrease at higher concentrations.

Most of the studies on the effects of thyroid-inhibiting drugs in amphibians have dealt with only one or two concentrations. Bruce and Parkes (1947), however, report that the metamorphosis of larvae of *Discoglossus pictus* is only slightly delayed when the animals are kept in 0.04% thiourea but is completely inhibited by 0.1% thiourea. Use of 0.2% and 0.5% solutions prevented metamorphosis but also affected the growth of the tadpoles adversely and resulted in a high mortality. Lynn (1948) found that larvae of *Eleutherodactylus ricordii* showed no effects on their growth or metamorphic pattern when treated with 0.001% thiourea, but were increasingly affected by concentrations of 0.005% and 0.05%. The thyroids of the larvae treated with 0.05% thiourea exhibited marked changes but those of animals from the two lower concentrations were not significantly affected. The work of Ratzersdorfer, Gordon and Charipper (1949), although it deals with a lizard rather than an amphibian, is also of interest in this connection for they found that high concentrations of thiourea when administered by injection produced less marked effects on the thyroid than did lower concentrations. These authors ascribed this result to (p. 23) "toxic reactions which tend to mask partially the goitrogenic effect." The nature of the toxic reactions involved is uncertain but it does seem likely that for any goitrogenic drug there is, for any specific animal, an optimal concentration above which less pronounced effects are obtained. This fact may account for the results reported by Adams (1946) who found that the thyroid of adult *Triturus viridescens* which had been kept in thiourea solutions for periods up to 86 days showed relatively slight changes. These newts were in two groups and had been treated with 0.033% and 0.066% thiourea during the first 28 days of the experiment but the concentrations were later increased in two steps so that for the last 44 days the concentrations were 0.528% and 1.056%. In the light of the present results it seems probable that these latter concentrations were above the optimal level and were, therefore, less effective than somewhat lower concentrations would have been.

Of the four different goitrogens tested in the present study, phenylthiourea appears to have the greatest potency. At a concentration of 0.005% this drug produced effects comparable to those obtained with 0.1% thiourea. Thiouracil at a concentration of 0.05% gave effects similar to those resulting from treatment with the same concentration of thiourea while allylthiourea at the same concentration, though its effect was delayed, seemed to produce more marked changes than thiourea. Arrangement of the drugs in decreasing order of effectiveness would thus be: phenylthiourea, allylthiourea, thiourea, thiouracil, with the last two, as far as the present evidence indicates, being approximately equivalent.

Although the relative potencies of a very large number of goitrogens have been tested for common laboratory mammals (Astwood, 1943; Astwood, Bissell and Hughes, 1945) there is very little information available concerning the relative effectiveness of these drugs in cold-blooded animals. Lynn's (1948) results with phenylthiourea and thiourea administered to larvae of the toad *Eleutherodactylus ricordii*, are in agreement with the present results in indicating a greater potency for the former substance. Gasche and Druey (1946) reported that allylthiourea is 10 to 20 times as effective as thiourea in inhibiting metamorphosis in Xenopus larvae and Koch (1948) also found indications of a high potency for allylthiourea in the inhibition of metamorphosis of *Rana temporaria* larvae. Thomas (1947) found that both phenylthiourea and allylthiourea show a greater effectiveness than thiourea when tested on *Rana pipens* tadpoles but he gives no information on the relative potency of the two first-named drugs. Because of differences in the methods of administration and in the criteria for effectiveness, the results of these studies, so far as they relate to the relative potencies of the goitrogens in question, are difficult to evaluate. It is clear that a detailed assay of the various goitrogens is necessary before any conclusions can be drawn concerning the relative potency of the drugs in amphibians.

The fact that long-continued treatment with thiourea results in decrease in the size and epithelial height of the thyroid was noted in the first investigations of the effects of goitrogen on amphibian material (Gordon, Goldsmith and Charipper, 1943, 1945). These authors found that *Rana pipiens* larvae treated with 0.033% thiourea for three weeks showed slightly enlarged and markedly activated thyroid glands. This condition persisted until about the seventh week of treatment but after this a "regression" occurred which resulted in a decrease in size of the gland as well as a marked change in its histology. Later study by Joel, D'Angelo and Charipper (1949) showed that this same phenomenon occurs in the adult frog after prolonged thiourea treatment and also that the gland can be reactivated by administration of thyrotrophic hormone. These authors, therefore, conclude that the thyroid "regression" is due to a failure in the production or the release mechanism of the thyrotrophic hormone or to a failure in both. It has been seen that in the present experiments a number of the treatments tested, notably those which produced histological evidence of early and maximal thyroid activation, resulted in histological changes indicating decreased thyroid activity after long-continued administration. Thus adult salamanders, like both larval and adult frogs, exhibit this phenomenon.

Since the thyroid activation and the later "regression" are presumed to result from changes in the level of thyrotrophic hormone produced by the pituitary gland, the pituitaries of the experimental animals were subjected to study. It was found that the pituitaries of animals whose thyroids showed well-defined signs of activation differed from those of normal animals in showing an increase in relative numbers of basophilic cells and a decrease in acidophils. In specimens whose thyroids were undergoing "regression" basophils were still predominant but there was evidence that some of these cells were undergoing degenerative changes, the cellular outlines becoming indistinct and the nuclei pycnotic.

There is an extensive literature on the possible localization of secretory function in the various cell types in the pituitary. Most of the evidence concerning the source of the thyrotrophic hormone is derived from studies of the effects of thyroidectomy upon the histology of the pituitary. When the thyroid gland is removed the pituitary responds by an increased production of thyrotrophic hormone and one would assume that the increased activity of the cells which secrete this hormone would be accompanied by some change in their appearance or relative numbers. The results of such investigations have been fully reviewed by Adams (1946) and will not be considered in detail here. While there is some disagreement, most of the work indicates that after thyroidectomy definite changes occur in the basophils. The changes most frequently reported are vacuolization of the cells and an increase in their number. There is considerable divergence of opinion as to whether any significant changes in the acidophils regularly occur.

With the discovery of the goitrogens as tools for carrying out "chemical thyroidectomy," several investigators turned to the use of these drugs as a means of obtaining further evidence concerning the source of the thyrotrophic hormone. Griesbach (1941) reported that the pituitaries of mammals given a goitrogenic diet exhibit a rapid increase in basophilic elements but that this increase, after reaching a maximum of 56 days, is followed by a return to a more normal condition. The time of return of the pituitary to normal coincided with the time of appearance of colloid in the hyperplastic thyroid glands and Griesbach considered this consistent with the hypothesis that the basophils are the source of the thyrotrophic hormone. Gasche and Druey (1946), who appear to be the only investigators who have previously studied the effect of goitrogens on amphibian pituitaries, refer only briefly to the fact that the pituitaries of *Xenopus laevis* larvae raised in thiourea exhibit the same changes in basophil cells as those which appear after thyroidectomy.

As has been seen, the present work is in agreement with most previous studies in that interference with the normal functioning of the thyroid gland was followed by an increase in the number of basophils in the anterior lobe of the pituitary. The results of long-continued treatment with goitrogens, however, do not agree with those reported by Griesbach. The "regression" which occurred in the thyroid was not accompanied by any return of the pituitary to a normal histological condition; instead, the pituitary showed increasing abnormality with signs of degeneration of some of the basophilic elements. This, however, does not seem surprising since the decreased thyroid activity is assumed to result from failure of the thyrotrophic hormone-producing cells. In fact, since the present results are in such accord with the changes reported by most authors following thyroidectomy, they may be interpreted as adding one more confirmation to the hypothesis that basophils of the anterior lobe of the pituitary are the sources of the thyrotrophic hormone. It must be pointed out, however, that there is another possible interpretation of the pituitary changes. Long-continued treatment with thiourea and similar substances may have direct effects upon the histology of the pituitary and the changes observed therefore may rest upon this basis rather than upon the over-stimulation of thyrotrophic hormone production resulting from thyroid inhibition. So far as the author is aware none of the studies of the results of administration of goitrogens have eliminated the possibility that these drugs might have selective effects upon certain cellular elements in the pituitary which after a time might cause degenerative changes in these elements. It is planned to study the pituitaries of a series of animals given simultaneous treatment with thyroxin and thiourea. Presumably the administration of thyroxin at an appropriate concentration would prevent any stimulation of the pituitary to over-production of thyrotrophic hormone and if any changes in the pituitary then occur such changes could be ascribed to direct effects of the thioura.

## SUMMARY

1. A study of the effect of various concentrations of four thyroid-inhibiting substances upon thyroid and pituitary glands in *Desmognathus fuscus brimleyorum* Stejneger has been made.

2. The optimal concentration for thiourea was found to be in the region of 0.01% to 0.3%.

3. The order of effectiveness of the goitrogens tested was phenylthiourea, allylthiourea, thiourea and thiouracil.

4. After long-continued treatment with concentrations of optimal efficiency, the thyroid showed histological evidence of a decrease in activity.

5. Pituitary preparations showed an increase in basophilia as a result of the treatment.

6. Pituitaries of animals which had shown decreased activity in the thyroid preparations exhibited an onset of degeneration in the basophilic elements.

## LITERATURE CITED

Adams, A. E., 1946. Variations in the potency of thyrotrophic hormone of the pituitary in animals. *Quart. Rev. Biol.*, **21**: 1–32.

Astwood, E. B., 1943. The chemical nature of compounds which inhibit the function of the thyroid gland. *J. Pharmacol. Exp. Therap.*, **78**: 78–79.

Astwood, E. B., 1949. Mechanisms of action of various antithyroid compounds. *Ann. N. Y. Acad. Sci.*, **50**: 419–443.

Astwood, E. B., A. Bissell and A. M. Hughes, 1945. The antithyroid activity of thiobarbital (5,5-diethyl-2-thiobarbituric acid). *Endocrinology*, **36**: 72–74.

Baumann, E., N. Metzger and D. Marine, 1944. Mode of action of thiourea on the thyroid gland of rabbits. *Endocrinology*, **34**: 44–49.

Blakstad, T. W., 1949. Depigmentation in *Rana temporaria* tadpoles as a result of methylthiouracil treatment. *J. Endocrinol.*, **6**: 23–27.

Bruce, H. M., and A. S. Parkes, 1947. Observations on *Discoglossus pictus* Otth. *Proc. Roy. Soc. Lond., Ser. B*, **134**: 37–56.

Charipper, H. A., and A. S. Gordon, 1947. The biology of antithyroid agents. *Vitamins and Hormones*, **5**: 273–316.

Delsol, M., 1948. Action du thiourcil sur la metamorphose de *Discoglussus pictus* Otth. *C. R. Soc. Biol., Paris*, **142**: 458–460.

Gasche, P., and J. Druey, 1946. Wirksamkeit schilddrüsenhemmender Stoffe auf die Xenopusmetamorphose. *Experientia*, **2**: 26–27.

Gordon, A. S., E. D. Goldsmith and H. A. Charipper, 1943. Effect of thiourea on the development of the amphibian. *Nature*, **152**: 504–505.

Gordon, A. S., E. D. Goldsmith and H. A. Charipper, 1945. The effect of thiourea on amphibian development. *Growth*, **9**: 19–41.

Griesbach, W. E., 1941. Changes in the anterior pituitary of the rat, produced by Brassica seed diet. *Brit. J. Exp. Path.*, **22**: 245–249.

Harms, J. W., 1949. Transplantation von Regenerationsgewebe in die vordere Augenkammer bei *Triton alpestris* und *cristatus* sowie Versuche über die Wirkung von Thioharnstoff bei *Xenopus laevis*. *Klin. Mb. Augenheilk.*, **114**: 298–308.

Hughes, A. M., and E. B. Astwood, 1944. Inhibition of metamorphosis in tadpoles by thiouracil. *Endocrinology*, **34**: 138–139.

Joel, T., S. A. D'Angelo and H. A. Charipper, 1949. The effect of thiourea on the thyroid gland of winter frogs (*Rana pipiens*) with some observations on the testis. *J. Exp. Zool.*, **110**: 19–31.

Koch, J., 1948. Über bei Wirkung substitutierter Thioharnstoffe auf die Metamorphose der Kaulquappe. *Biochem. Zeitschr.*, **318**: 515–520.

Lever, J., 1951. On the influence of antithyroid substances on the hormone production of the thyroid gland. *Experientia*, **7**: 201–207.

Lynn, W. G., 1948. The effects of thiourea and phenylthiourea upon the development of *Eleutherodactylus ricordii*. *Biol. Bull.*, **94**: 1–15.

Lynn, W. G., and Sr. A. De Marie, 1946. The effect of thiouracil upon pigmentation in the tadpole. *Science*, **103**: 31.

Lynn, W. A., and H. E. Wachowski, 1951. The thyroid gland and its function in cold-blooded vertebrates. *Quart. Rev. Biol.*, **26**: 123–168.

Mixner, J. P., E. P. Reineke and A. W. Turner, 1944. Effect of thiouracil on the thyroid gland of the chick. *Endocrinology*, **34**: 168–174.

PEARSE, A. G., 1950. Differential stain for the human and animal anterior hypophsis. *Stain Tech.*, **25**: 95.

PITT-RIVERS, R., 1950. Mode of action of antithyroid compounds. *Physiol. Rev.*, **30**: 194–205.

RATZERSDORFER, C., A. S. GORDON AND H. A. CHARIPPER, 1949. The effects of thiourea on the thyroid gland and molting behavior of the lizard, *Anolis caroliensis*. *J. Exp. Zool.*, **112**: 13–27.

THOMAS, A., 1947. Effects of some thyroid-inhibitors upon the development of *Rana pipiens* tadpoles. *Anat. Rec.*, **99**: 63.

TROTTER, W. R., 1949. Some recent developments in the pharmacology of the antithyroid compounds. *J. Phar. Pharmacol.*, **1**: 65–77.

Vol. 104, No. 3 June, 1953

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

---

# CELL DIVISION INHIBITION OF ARBACIA AND CHAETOPTERUS EGGS AND ITS REVERSAL BY KREBS CYCLE INTERMEDIATES AND CERTAIN PHOSPHATE COMPOUNDS[1, 2]

ROBERT C. BARNETT[3]

*The University of Chicago, Department of Physiology, Chicago, Illinois, and the Marine Biological Laboratory, Woods Hole, Mass.*

It has been demonstrated that anoxia (Loeb, 1895), cyanide (Lyon, 1902), azide (Clowes and Krahl, 1939), and dinitrophenol (DNP) (Clowes and Krahl, 1936) inhibit the cleavage of *Arbacia punctulata* eggs. These metabolic inhibitors are known to interfere with hydrogen transport (Stannard and Horecker, 1948) and/or phosphorylation (Reiner and Spiegelman, 1947). Because "high energy phosphate" ($\sim$P) is equilibrated with the adenosine triphosphate (ATP) within the cell (Green and Colowick, 1944), it was considered possible that the inhibition of cleavage, produced by the above agents, might be reversed by addition of ATP. Further, because current ideas relate the oxidative formation of $\sim$P to the functioning Krebs cycle (Lipman, 1941), inhibition of the cycle by malonate might inhibit cleavage because of $\sim$P lack. If the general scheme of $\sim$P generation applied to *Arbacia*, relief of the malonate division inhibition would be effected by succinate, a substance which competes with malonate for succinic dehydrogenase; fumarate, an intermediate of the Krebs cycle beyond the point of action of malonate (Potter and Dubois, 1943); and ATP, the postulated critical product of the cycle (Lipman, 1946).

Two reasons prompted an extension of the experiments to include *Chaetopterus*, as well as *Arbacia*, eggs: (1) *Arbacia* eggs show an increase, and *Chaetopterus* eggs a decrease, in oxygen consumption at the time of initiation of cell division (Whitaker, 1932); (2) if *Arbacia*, an Echinoderm, and *Chaetopterus*, an Annelid, exhibit similar reactions to malonate, it could indicate a general importance of the Krebs cycle and ATP to cell division in aerobic organisms.

---

[1] The author would like to express his sincere gratitude to Dr. J. O. Hutchens, Dr. D. L. Harris and Dr. J. Z. Hearon for their encouragement and constructive criticism. Dr. G. H. A. Clowes was kind enough to make the Eli Lilly Laboratories at Woods Hole available for part of this work. Grateful acknowledgment is made to Manfred Brust for technical assistance.

[2] Completed in part while Fellow of the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

[3] Present address: Department of Physiology, University of Texas—Medical Branch, Galveston, Texas.

## METHODS

*Arbacia punctulata* and *Chaetopterus pergamentaceus* eggs were washed in sea water and adjusted to 0.1 to 0.2 per cent by volume by taking a small aliquot of the egg suspension and centrifuging it at 1000 times gravity in duplicate hematocrit tubes. From the egg volume the number of eggs present in each experimental vessel was calculated (Harvey, 1932; Krahl, 1950). A second aliquot of eggs was fertilized and the per cent cleavage noted at the end of one hour as a test of both egg and sperm viability before use. Egg suspensions were used only if they showed 90 to 100 per cent cleavage. The fertilized eggs were added to the experimental vessels after 25 minutes (*Arbacia*) or 40 minutes (*Chaetopterus*).

In low oxygen tension experiments, mixtures of air and nitrogen were prepared by displacement of water in a calibrated glass bottle (Umbreit *et al.*, 1949). The bottle was connected by rubber tubing to serially arranged double-arm Warburg vessels which contained, in the main compartment, fertilized eggs and reversing agent or NaCl. The rate of gassing was maintained approximately constant by regulating the rate of bubble evolution by means of a screw clamp placed at the end of the last tube which dipped below the surface of the water bath. The gas mixture was passed through the serially connected vessels for 30 minutes with shaking at an amplitude of six centimeters and rate about 120 per minute. At the end of the gassing period the vessels were closed and the shaking continued for an additional two hours. The control differed from the experimental vessels only in the substitution of air for the gas mixture. The disadvantage of a serially connected system is the possibility of unequal gassing of the individual flasks. To minimize this disadvantage, the order of the flasks was varied from experiment to experiment. To insure adequate removal of air, the volume of flushing gas used was 20 times the volume of the empty system.

When azide, cyanide, and DNP[4] were used as division-inhibiting agents, the experiments were conducted in air-filled closed vessels to which 3.3 mg. per ml. of glycylglycine buffer was added at pH 8.2. No buffer was required with malonate and its reversing agents, succinate and fumarate, because the latter solutions were adjusted to pH 8.2 with solid sodium hydroxide. Two types of experiments were performed when malonate was used to inhibit cell division. In some experiments solid sodium hydroxide was added to malonic acid dissolved in sea water which produced a hypertonic solution. In other experiments solid sodium hydroxide was added to malonic, fumaric or succinic acid dissolved in distilled water to produce a solution isotonic with sea water. Experimental temperature varied between 22–27° C., but within a single experiment the temperature was constant to ± 0.1° C.

In all instances at the conclusion of the experiment (two to three hours) egg cleavage was stopped with formaldehyde and the number of divisions per egg in each vessel was calculated according to the method of Smith and Clowes (1924). In this calculation 100 eggs are counted and the cleavage stage of each egg is recorded as follows: no divisions, one cell; one division, two cell; two divisions, four cell; etc. The sum of the number of divisions divided by 100 yields a number designated as the number of divisions per egg.

---

[4] The DNP was a purified sample obtained through the kindness of Dr. G. H. A. Clowes of the Eli Lilly Laboratories, Woods Hole, Mass.

## EXPERIMENTAL RESULTS

### *Interference with the hydrogen transport system near its terminus*

Graphically presented in Figure 1 are the results of typical experiments in which low oxygen tension was used to inhibit cleavage of *Arbacia* eggs. It is seen that in all instances ATP stimulated cell division of the inhibited eggs. Further, the added ATP completely reversed the division inhibition provided the latter was not greater than 50 per cent. Figure 2 shows that adenylic acid (AA), hydrolyzed ATP (HATP), or the ~ P compound, inorganic triphosphate (ITP), were not effective in reversing the division inhibition.

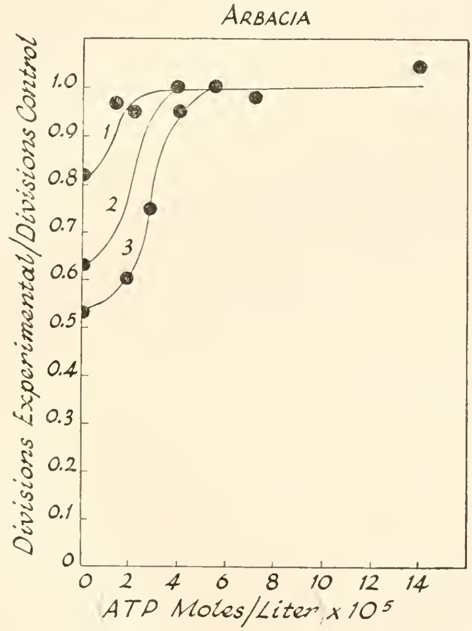


FIGURE 1. The reversal effect of ATP on low oxygen tension-inhibited *Arbacia* eggs. Rate of oxygen consumption as per cent of control: (1) 75; (2) 60; (3) 50. Experimental duration 100 minutes.

Because added ATP completely reversed low oxygen tension inhibition of *Arbacia* egg cleavage, it was thought a similar reversal might be obtained when cyanide or azide were used as inhibiting agents. The results with sodium azide are presented in Figure 3. The results with sodium cyanide are presented in Figure 4. It is seen that the inhibition of cleavage in *Arbacia* eggs is:

(1) Reversed when ATP is added to cyanide-inhibited cells.
(2) Not reversed when ATP is added to azide-inhibited cells.
(3) Not reversed when hydrolyzed ATP is added to either cyanide- or azide-inhibited cells.

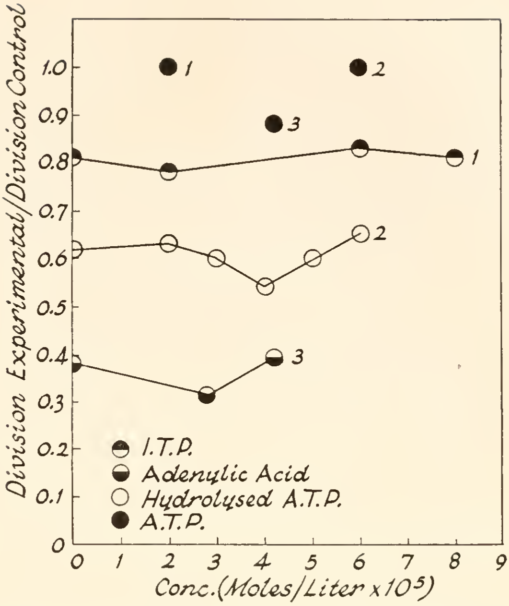


FIGURE 2. The effect of ATP, ITP, AA and HATP on low oxygen tension—inhibited *Arbacia* eggs. The corresponding numbers are the results obtained with the same batch of eggs. Experimental period 100 minutes.

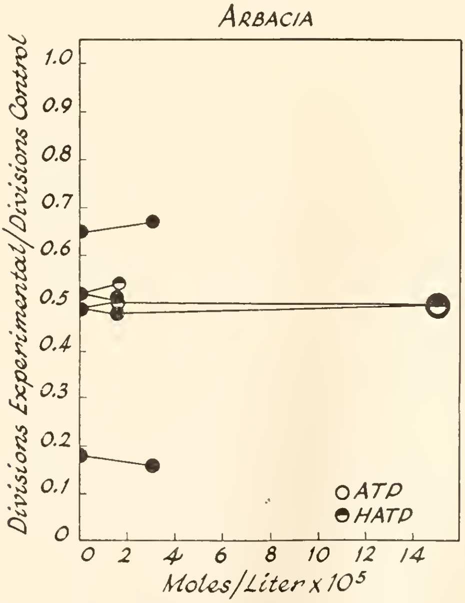


FIGURE 3. The effect of ATP and HATP on azide-inhibited *Arbacia* eggs. With increasing inhibition the concentration of $NaN_3$ was: 1.35, 1.25, 2.7 × $10^{-3}$ $M$ sodium azide, and the experimental time was 180, 100 and 180 minutes, respectively.

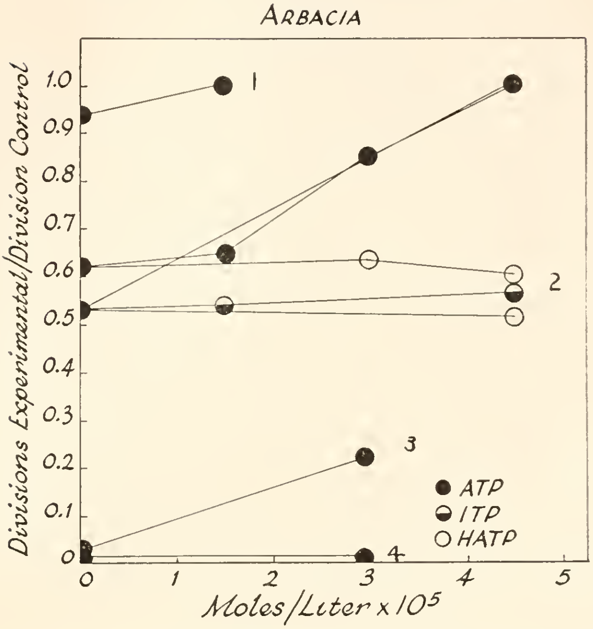


FIGURE 4. ATP, ITP and HATP effect on cyanide-inhibited *Arbacia* eggs. Cyanide concentration (1) 2; (2) 6; (3) 8; (4) $16 \times 10^{-5}$ *M*. Experimental time 100 minutes.

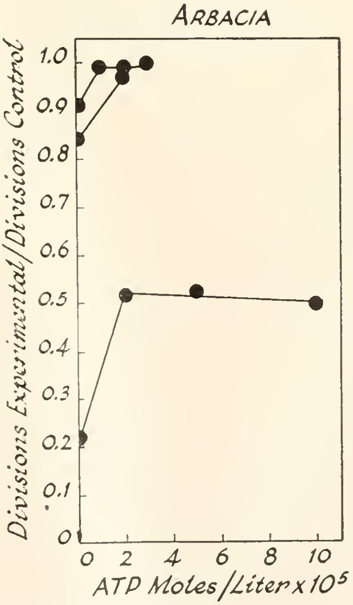


FIGURE 5. ATP effect on DNP-inhibited *Arbacia* eggs. Concentration of DNP with increasing amount of inhibition: 0.4, 0.8 and $1.6 \times 10^{-5}$ *M*. Experimental time 100 minutes.

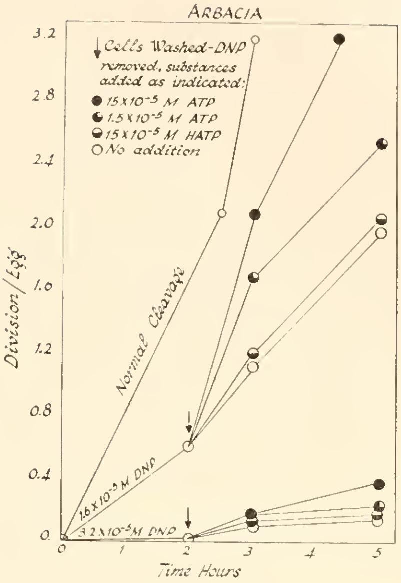


Figure 6. Recovery of *Arbacia* eggs following DNP inhibition.

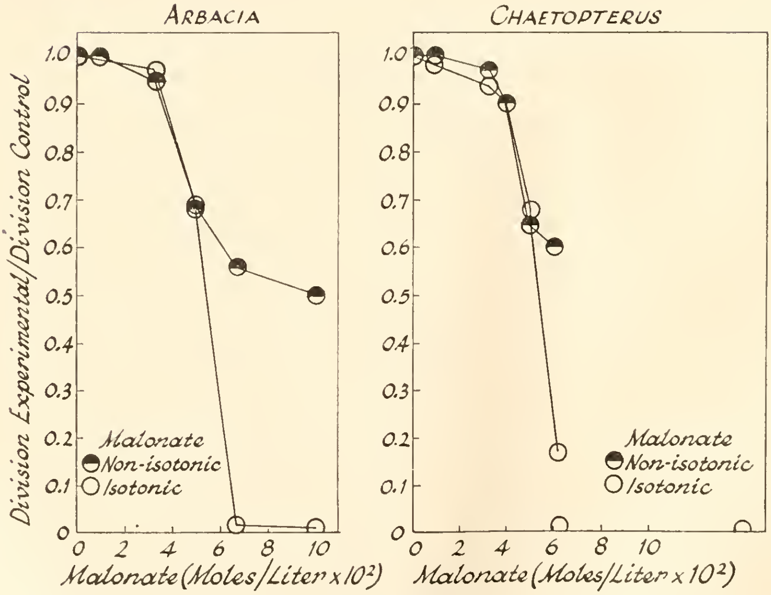


Figure 7. The inhibitory effect of isotonic and non-isotonic malonate on the cleavage of *Arbacia* and *Chaetopterus* eggs.

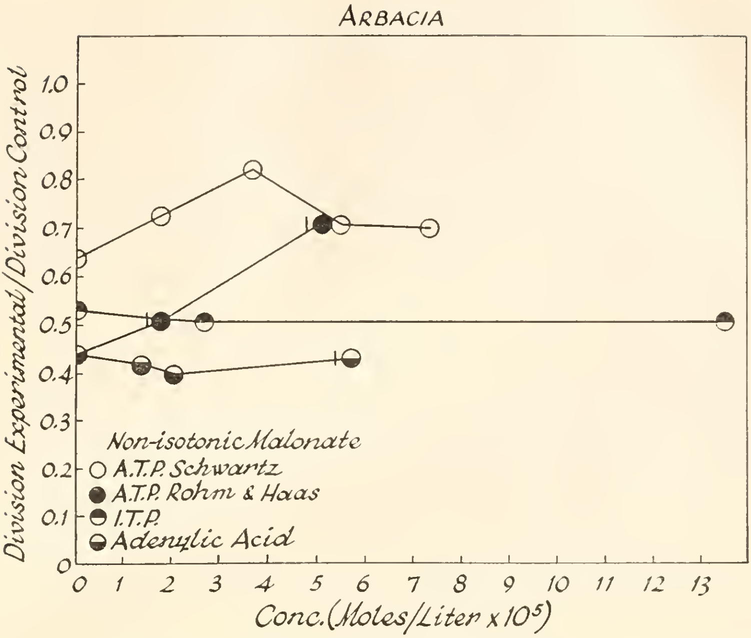


FIGURE 8. The effect of ATP, ITP and AA on non-isotonic malonate-inhibited *Arbacia* eggs. Experimental time 100 minutes.

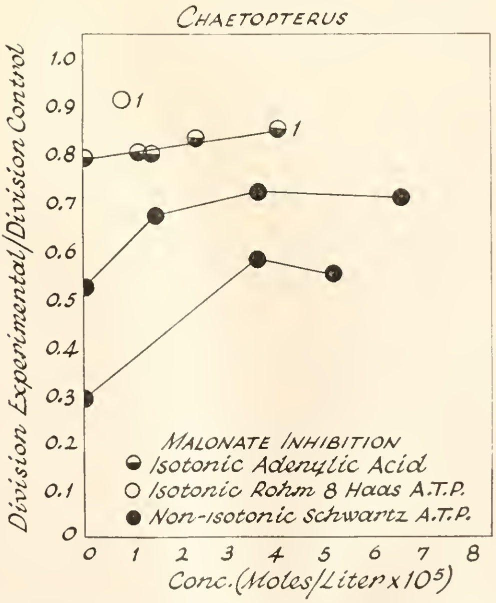


FIGURE 9. ATP and AA effect on isotonic and non-isotonic malonate-inhibited *Chaetopterus* eggs. The number indicates same experiment and same batch of eggs. Experimental time 100 minutes.

Azide, in addition to inhibiting cytochrome oxidase, is known to inhibit transphosphorylation and ATPase activity (Meyerhof, 1945). The observation that ATP was unable to reverse the azide inhibition of cleavage in *Arbacia* eggs suggested testing DNP, a substance believed to dissociate phosphorylation from oxidation (Loomis and Lipman, 1948). Figure 5 presents the data of the effect of ATP on DNP-inhibited *Arbacia* eggs. It will be noted that ATP was able partially to reverse the division inhibition. Figure 6 shows the recovery of *Arbacia* eggs after DNP inhibition. During the recovery period ATP and hydrolyzed ATP were added. The experiment shows that ATP stimulated cleavage after the removal of DNP.

### *Malonate inhibition of Arbacia and Chaetopterus eggs*

The experimental findings obtained with malonate as the cell division inhibitor are presented in Figure 7. Figures 8 and 9 present the effect of added ATP, ITP, and AA on the malonate-inhibited *Arbacia* and *Chaetopterus* eggs, respectively. Figure 10 presents the effect of fumarate and succinate on malonate-inhibited eggs.

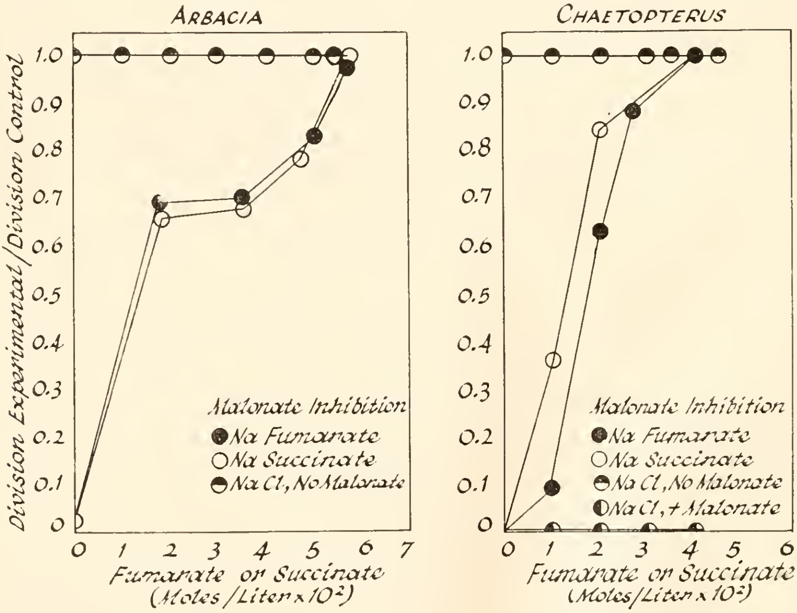


FIGURE 10. The complete reversal of malonate-inhibited *Arbacia* and *Chaetopterus* eggs by succinate and fumarate using isotonic $10^{-1}$ *M* (*Arbacia*) and $6 \times 10^{-2}$ *M* isotonic malonate (*Chaetopterus*).

It is seen that malonate completely inhibits cleavage of both *Arbacia* and *Chaetopterus* eggs in isotonic solution. It is noted further that *Arbacia* and *Chaetopterus* eggs are 50 per cent protected against malonate by hypertonicity. At the concentrations tested, succinate and fumarate completely, and ATP incompletely, reverse the malonate inhibition. Complete reversal of the malonate inhibition was not obtained by added ATP at the concentrations which completely reversed division inhibition due to reduced oxygen tension.

## Discussion and Conclusions

Sea urchin eggs fail to divide at low oxygen tensions. From this observation Loeb (1895) and Warburg (1914) postulated that extra energy, dependent upon oxygen, was necessary for division of these eggs. Thus, if oxidative energy is involved in cell division of *Arbacia* eggs, then a decrease in the oxygen tension could inhibit division in at least two ways:

(1) Decrease in hydrogen transport, not associated with phosphorylation.
(2) Decrease in $\sim$ P formation.

An experiment performed by Korr (1939) suggests that a decrease in hydrogen transport *per se* is not primarily involved in the inhibition of *Arbacia* egg division. He added cyanide to respiring *Arbacia* eggs which resulted in a decrease in the oxygen consumption and an inhibition of division. The respiration was restored to normal by addition of methylene blue but division remained inhibited. Methylene blue is known to by-pass the dehydrogenase-cytochrome hydrogen transport system which has been shown to generate $\sim$ P (Lehninger, 1949). Consequently, these results have been interpreted as indicating that division fails because of insufficient $\sim$ P formation.

Whether reduced oxygen tension is able to decrease $\sim$ P generation and subsequently inhibit cell division depends upon the relative amount of $\sim$ P formed aerobically and anaerobically by the cell. For example, frog eggs show a very high rate of glycolysis (Barth and Jaeger, 1947). Reduction of the oxygen consumption of these eggs by cyanide or reduced oxygen tension has no effect upon division or the concentration of easily-hydrolyzed phosphate (interpreted as $\sim$ P). Addition of azide to the respiring frog eggs not only reduces the oxygen consumption and the concentration of the easily-hydrolyzable phosphate but also inhibits division. Thus, reducing the oxygen consumption of frog eggs has no effect on division; whereas, a reduction of the concentration of easily-hydrolyzable phosphate inhibits their division.

Unlike frog eggs, division of *Arbacia* eggs is inhibited when the oxygen tension is reduced, and it is not known whether the $\sim$ P concentration of the egg-cell is reduced under these conditions. It is apparent, however, from results here presented that intact ATP is able to support division of *Arbacia* eggs when their division has been inhibited by low oxygen tension. These results are indicative that division failed because of insufficient ATP and/or substances or systems dependent upon ATP. There is a linear relation between oxygen tension and division in the range where oxygen tension influences division (Clowes and Krahl, 1939). Hence, if division is dependent upon $\sim$ P, it might be expected that the amount of ATP added as $\sim$ P would be at least equivalent to that which would have been formed had the oxygen consumption remained undisturbed. The following simple calculation shows that the micromoles of $\sim$ P added as ATP is approximately ten times that calculated to be required. This calculation constitutes no proof that the ATP added was used as an energy source necessary for division. Further experiments are in progress to evaluate this point.

Normal dividing and respiring *Arbacia* eggs consume 2.4 mm.$^3$ oxygen per 10 mm.$^3$ egg hours (Hutchens *et al.*, 1942). Because 46,500 eggs occupy a volume of 10 mm.$^3$ (Harvey, 1932), the oxygen consumed per egg hour is $5.2 \times 10^{-5}$ mm.$^3$. Division is normal and 50 per cent inhibited when the oxygen consumption of the *Arbacia* egg is reduced to 80 per cent and 50 per cent, respectively (Clowes and Krahl, 1939). Thus, $1.42 \times 10^{-6}$ microatoms of oxygen per egg hour is the difference between the amount of oxygen consumed by normal and 50 per cent division-inhibited eggs. This oxygen consumption difference may be converted to micromoles of $\sim$ P on the assumption that three micromoles of $\sim$ P are produced per microatom of oxygen consumed (Ochoa, 1943). The result is $4.26 \times 10^{-6}$ micromoles of $\sim$ P per egg hour.

The amount of ATP added to 930 eggs was $4.1 \times 10^{-5}$ mM, or $2.05 \times 10^{-5}$ mM of ATP per egg hour for the two-hour experimental period. ATP contains two $\sim$ P bonds (Lohman, 1938). Therefore, the amount of $\sim$ P added is 4.4 $\times 10^{-5}$ micromoles per egg hour as compared with $4.26 \times 10^{-6}$ micromoles $\sim$ P per egg hour calculated from the oxygen consumption difference. These calculations indicate that the micromoles of $\sim$ P added as ATP are approximately ten times the amount calculated to be required.

The calculation presented is at least consistent with the concept that ATP acts to supply $\sim$ P to anoxia-inhibited eggs. However, that something in addition to ATP is necessary for division is indicated by the experiments in which ATP was added to malonate- and DNP-inhibited eggs. In both these instances, added ATP stimulated the inhibited eggs, but in no instance was the division rate re-established to normal. Greater reversal might have been obtained had higher concentrations of ATP been used. However, Runnström and Kriszat (1951) found a maximum of 40 per cent reversal when 100 times the concentration of ATP was added to DNP-inhibited sea urchin eggs.

It has been suggested (Runnström and Gustafson, 1951) that ATP could affect cell division by influencing cytoplasmic viscosity, similar to the effect of ATP on actomyosin threads and solutions. It is well known (Heilbrunn, 1943) that at the time of division there is a marked decrease in protoplasmic viscosity, and Runnström and Kriszat (1950) have shown that ATP added to unfertilized eggs decreases the cellular cytoplasmic viscosity. To test the possibility that added ATP might function in cell division as a substrate for an actomyosin-like protein, ITP, Pyro. phosphate (Pyro. phos.), and ATP were added to division inhibited eggs. These reagents have been shown to contract actomyosin threads and to decrease the viscosity of actomyosin solutions (Biro and Straub, 1949). As previously noted, of these reagents only ATP was effective in promoting division of the inhibited eggs. These results may indicate: (1) that ITP and Pyro. phos. were not accessible to the division mechanism; (2) that the egg actomyosin-like protein does not react with these substances; or (3) that the function in division of added ATP is not to support an actomyosin-like reaction. Further experiments are in progress to evaluate these points.

To summarize the results presented, the action of an inhibitor which interferes with hydrogen transport (cyanide and low oxygen tension) can be completely reversed by added ATP, provided the inhibition is not greater than 50 per cent. If either ATP utilization or the generation of metabolic intermediates are interfered with (azide and DNP), ATP alone cannot make up the deficit. The fact that malonate is able completely to inhibit division of *Arbacia* and *Chaetopterus* eggs, and succinate and fumarate are able completely to reverse this inhibition, is strong evidence that division is intimately dependent upon a functioning Krebs cycle. Further, because ATP was unable to reverse completely the division inhibition due to the action of malonate, the function of the Krebs cycle in division may not be completely accounted for on the basis of ~ P generation.

## SUMMARY

Division of *Arbacia* eggs was inhibited by low oxygen tension, cyanide, azide, dinitrophenol (DNP) and malonate. Various high and low energy phosphate compounds, and succinate and fumarate were added to the inhibited eggs. The results were extended to malonate-inhibited *Chaetopterus* eggs and the effects of succinate and fumarate were noted.

1. Addition of $10^{-5}$ to $10^{-4}$ $M$ ATP completely reversed *Arbacia* egg division inhibition produced by cyanide and low oxygen tension. ATP incompletely reversed inhibitions caused by DNP and malonate. The maximum division stimulations were: with low oxygen tension and cyanide, division was increased from 50 per cent to 100 per cent of the control value; with $1.6 \times 10^{-5}$ $M$ DNP, division was increased from 23 to 51 per cent; with $7.5 \times 10^{-2}$ $M$ malonate, division was increased from 44 per cent to 71 per cent. With *Chaetopterus* eggs, the maximum stimulation obtained in the presence of 0.1 $M$ malonate was from 28 per cent to 58 per cent of the control value.
2. Adenylic acid, hydrolyzed ATP, inorganic phosphate, and the ~ P compounds, inorganic triphosphate and pyrophosphate, did not stimulate division of *Arbacia* eggs when inhibited by any of the above agents.
3. Division of *Arbacia* and *Chaetopterus* eggs was completely inhibited by $10^{-1}$ to $10^{-2}$ $M$ malonate. The malonate effect was completely reversed, in both egg types, by the addition of $5.7 \times 10^{-2}$ $M$ succinate or fumarate.
4. Azide inhibition was not relieved by added ATP or any of the other phosphorylated compounds tested.
5. These results suggest, in *Arbacia* and *Chaetopterus* eggs, that division is intimately related to a functioning Krebs cycle, the function of which cannot be completely accounted for on the basis of ~ P production.

## LITERATURE CITED

BARTH, L. G., AND L. JAEGER, 1947. Phosphorylation in the frog's egg. *Physiol. Zool.*, **20**: 133–146.

BIRO, A. K. S., AND F. B. STRAUB, 1949. The interaction between actomyosin and polyphosphates. *Hung. Acta. Physiol.*, **2**: 84–92.

CLOWES, G. H. A., AND M. E. KRAHL, 1936. Studies on cell metabolism and cell division. I. On the relation between molecular structures, chemical properties, and biological activities of the nitrophenols. *J. Gen. Physiol.*, **20**: 145–171.

CLOWES, G. H. A., AND M. E. KRAHL, 1939. Studies on cell metabolism and cell division. III. Oxygen consumption and cell division of fertilized sea-urchin eggs in the presence of respiratory inhibitors. *J. Gen. Physiol.*, **23**: 401–411.

GREEN, A. D., AND S. P. COLOWICK, 1944. Chemistry and metabolism of the compounds of phosphorous. *Ann. Rev. Biochem.*, **13**: 155–186.

HARVEY, E. N., 1932. Physical and chemical constants of the egg of the sea-urchin, *Arbacia punctulata*. *Biol. Bull.*, **62**: 141–154.

HEILBRUNN, L. V., 1943. An outline of general physiology. W. B. Saunders and Co., Philadelphia. 2nd Edition.

HUTCHENS, J. O., A. K. KELTCH, M. E. KRAHL AND G. H. A. GLOWES, 1942. Studies on cell metabolism and cell division. VI. Observations on the glycogen content, carbohydrate consumption, lactic acid production, and ammonia production of eggs of *Arbacia punctulata*. *J. Gen. Physiol.*, **25**: 717–731.

KORR, I. M., 1939. *Cold Spring Harbor Symp. Quan. Biol.*, **7**: 120 (Comment).

KRAHL, M. E., 1950. Metabolic activities and cleavage of eggs of the sea-urchin, *Arbacia punctulata*: a review, 1932–1949. *Biol. Bull.*, **98**: 175–217.

LEHNINGER, A. L., 1949. Esterification of inorganic phosphate coupled to electron transport between dihydrodiphosphopyridine nucleotide and oxygen II. *J. Biol. Chem.*, **181**: 625–644.

LIPMAN, F., 1941. Metabolic generation and utilization of phosphate bond energy. *Advances in Enzymology*, **1**: 99–162.

LIPMAN, F., 1946. Metabolic process patterns. Currents in biochemical research. Ed. by A. D. Green. Interscience Pub. Inc., New York.

LOEB, J., 1895. Untersuchungen über die physiologischen Wirkungen des Sauerstoffmangels. *Archiv. f. Ges. Physiol.*, **62**: 249–294.

LOHMAN, K., 1938. Konstitution der Adenylpyrophosphorsaüre und Adenosindiphosphorsaüre. *Biochem. Zeit.*, **282**: 120–123.

LOOMIS, W. F., AND F. LIPMAN, 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. *J. Biol. Chem.*, **173**: 807–808.

LYON, E. P., 1902. Effects of potassium cyanide and of lack of oxygen upon the fertilized eggs and the embryos of the sea-urchin (*Arbacia punctulata*). *Amer. J. Physiol.*, **7**: 56–75.

MEYERHOF, O., 1945. The origin of the reaction of Harden-Young in cell-free alcoholic fermentations. *J. Biol. Chem.*, **157**: 105–119.

OCHOA, S., 1943. Efficiency of aerobic phosphorylation in cell-free heart extracts. *J. Biol. Chem.*, **151**: 493–505.

POTTER, V. R., AND K. P. DUBOIS, 1943. Studies on the mechanism of hydrogen transport in animal tissues. VI. Inhibition studies with succinic dehydrogenase. *J. Gen. Physiol.*, **26**: 391–404.

REINER, J. M., AND S. SPIEGELMAN, 1947. The mechanism of inhibition of carbohydrate assimilation by dinitrophenol and azide. *J. Cell. Comp. Physiol.*, **30**: 347–357.

RUNNSTRÖM, J., AND T. GUSTAFSON, 1951. Developmental physiology. *Ann. Rev. Physiol.*, **13**: 57–74.

RUNNSTRÖM, J., AND G. KRISZAT, 1950. On the influence of ATP on the fertilization and segmentation of the sea urchin egg, *Stronglyocentrotus lividus*. *Exp. Cell Res.*, **1**: 497–499.

RUNNSTRÖM, J., AND G. KRISZAT, 1951. Some effects of adenosine triphosphate on the cytoplasmic state, division, and development of the sea-urchin egg. *Trans. N. Y. Acad. Sci.*, **13**: 162–164.

SMITH, H. W., AND G. H. A. CLOWES, 1924. The influence of carbon dioxide on the velocity of division of marine eggs. *Amer. J. Physiol.*, **68**: 183–202.

STANNARD, J. N., AND B. L. HORECKER, 1948. The *in vitro* inhibition of cytochrome oxidase by azide and cyanide. *J. Biol. Chem.*, **172**: 599–608.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER, 1949. Manometric techniques and tissue metabolism. Burgess Publishing Co., Minneapolis.

WARBURG, O., 1914. Zellstruckture und Oxydationsgeschwindigkeit nach Versuchen am Seeigelei. *Archiv. f. Ges. Physiol.*, **158**: 189–208.

WHITAKER, D. M., 1932. On the rate of oxygen consumption by fertilized and unfertilized eggs. *J. Gen. Physiol.*, **16**: 475–495.

# ENDOCRINE CONTROL OF METABOLISM IN THE LAND CRAB, GECARCINUS LATERALIS (FRÉMINVILLE). I. DIFFERENCES IN THE RESPIRATORY METABOLISM OF SINUSGLANDLESS AND EYESTALKLESS CRABS[1, 2]

DOROTHY E. BLISS

*The Biological Laboratories, Harvard University and Radcliffe College,*
*Cambridge 38, Massachusetts*

Long ago eyestalk removal was observed by Zeleny (1905) to induce molting in *Uca* and by Megušar (1912) to reduce the length of the intermolt interval of *Astacus.* Many years later Abramowitz and Abramowitz (1938, 1940), Hanström (1939), Brown and Cunningham (1939), Smith (1940) and Kleinholz and Bourquin (1941) also reported compression of the intervals between successive molts in a variety of eyestalkless crustaceans. Revival of interest in the crustacean eyestalk as a molt-inhibiting center may be attributed to the discoveries in the meantime that the eyestalk contained (1) chromatophorotropically active materials (Koller, 1925, 1927; Perkins, 1928), and (2) two organs, the sinus gland and the x-organ, which gave cytological evidence of secretory activity (Hanström, 1931, 1933). Subsequent studies, primarily by Brown and his students (see Brown, 1935, 1940), yielded considerable evidence favoring the sinus gland as the principal source of these chromatophore-activating materials.

The activity of the sinus gland did not seem to be confined to chromatophore regulation. Kleinholz (1934, 1936) clearly demonstrated the response of crustacean retinal pigments to eyestalk extract, thus confirming the suggestions of Welsh (1930a, 1930b) that there is hormonal control of these pigments. Later work by Welsh (1939, 1941) pointed directly toward the sinus gland as the agent responsible. On the basis of evidence offered by Brown and Cunningham (1939), Scudamore (1942, 1947), Kyer (1942) and Bauchau (1948a), molting was added to the list of processes thought to be controlled by the sinus gland.

It was realized, however, that since the concept of hormonal regulation by the sinus glands was based to a large extent upon effects of total eyestalk removal, the critical experiment remained to be done, namely, removal of sinus glands alone. A method devised by Brown (1942) produced in sinusglandless crayfish certain responses similar to those which characterize eyestalkless animals. A removal technique, by which injury to other eyestalk tissues was minimized, was designed and employed by Kleinholz (1947). He found (1948, 1949a, 1949b) that the

---

[1] This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service and, in addition, by a Fellowship from the Bermuda Biological Station and an Edward L. Mark Fellowship for research at the Bermuda Biological Station.

[2] These materials constitute a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from Radcliffe College.

proximal pigments of the crayfish retina behaved normally after sinus gland removal. Extra-sinusglandular sources of retinal pigment hormone had not been conclusively demonstrated, since there remained the possibility, according to Kleinholz, that the pigments could also act as independent effectors. Nevertheless, here was an indication that, in one respect at least, a crustacean without its sinus glands could still be normal.

With the application by recent workers of the Kleinholz sinus gland removal technique or its various modifications, the mystery of the sinus gland has been clarified. Most of this work has been reported only in abstracts (Bliss, 1951; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951a, 1951b, 1952a; Travis, 1951b; Welsh, 1951) or in unpublished theses (Frost, 1948; Havel, 1949; Saloum, 1951; Travis, 1951a; Bliss, 1952; Passano, 1952b). The present paper summarizes physiological evidence in support of morphological observations and interpretations already presented (Bliss and Welsh, 1952). Each sinus gland of a crab should now be conceived as a storage-release center for secretory material synthesized within cells of a diffuse neurosecretory system, involving the eyestalk ganglia, the brain and perhaps the thoracic ganglionic mass. Metabolic data obtained by Kleinholz and co-workers, coupled with morphological observations by Mr. James B. Durand, Jr. of Harvard University (personal communication), suggest that a similar relationship, demonstrable in crayfish, characterizes the Macrura as well.

## MATERIALS AND METHODS

Specimens of *Gecarcinus lateralis* (Fréminville) were available in large numbers in Bermuda where these studies were commenced. During the major part of the work specimens were shipped periodically to Cambridge from Bermuda. Laboratory stocks were maintained at room temperature in sand-filled cement tanks, divided by wire partitions into sections suitable for 15 to 20 crabs each. During observation of a given crab, the animal was isolated in a rectangular gallon glass jar to which had been added sand and a small finger bowl or, in the case of eyestalkless crabs, a Petri dish of sea water or tap water. The crabs were observed to sit in the water and occasionally, by use of a large claw, to drink. Peanuts, lettuce and carrots were accepted by the crabs, peanuts being preferred. Mortality both during air shipment and during maintenance was very low.

### *Surgery*

The crab was cooled in the refrigerator for about an hour before sinus gland removal. It was then supported by plasticene in a cardboard holder which in turn lay, surrounded by ice, in a Büchner funnel. Rubber sheeting was laid over the crab's back and more ice was placed on the sheeting. The Büchner funnel was so mounted in a wooden table that the funnel top was flush with the table top (see Williams, 1946). Ice melt-water ran from the funnel into a collecting pan beneath the table.

Eyestalks were ligatured with coarse surgical cotton thread. By means of a dental burr (1.5 mm. diameter), which was mounted in a handpiece attached to a foot-regulated rheostat, a hole was drilled in the mid-dorsal portion of the eyestalk as it lay in its orbit. After the hypodermis had been moved aside and the connective tissue sheath slit, the blue-white sinus gland was extracted by watchmakers' forceps and a small piece of plastic cover slip was sealed with paraffin into the hole. The ligature was then released. The second eyestalk was treated in the same manner.

Eyestalk removal was also preceded by cooling of the crab in the refrigerator and followed by packing of the eyestalk stub with fibrin foam (Cutter Laboratories) or Gelfoam (Upjohn Company). Mortality after either operation was negligible when the crabs were cooled sufficiently. Eyestalk removal without previous cold anesthesia was often fatal, as were operations performed when crabs had remained over an hour in the refrigerator.

### *Measurement of respiratory rate and respiratory quotient*

For respiratory measurements use was made of a volumetric respirometer, the design of which was suggested to the writer by Dr. P. F. Scholander. All plastic and metal portions of this instrument were machined by Mr. John R. Andrews of Randolph, Massachusetts. Of similar principle is the respirometer employed by Flemister and Flemister (1951). Figure 1A shows the essential features of the instrument used by the writer.

The edges of a jar (1) of 450 cc. or 250 cc. capacity have been ground with carborundum so that, when greased and clamped, they make an air-tight seal with a ground plastic cover (2), cut from 1/2-inch Lucite. Dead air space in the animal chamber (1) has been reduced by the addition of paraffin (3). The animal chamber is clamped by a quarter-inch brass crossbar (4), which slides up and down on two threaded brass uprights mounted on either side of a Lucite platform (5). The platform is supported by a strip of brass (6). One arm of a plastic manometer (7), designed by Scholander (1949), is connected to the animal chamber. The other arm of the manometer leads to a thermobarometer (8), a bottle having a capacity of 250 cc. and containing a small amount of water. Manometer fluid, devised by Dr. Howard A. Schneiderman of Harvard University, consists of a 1:200 dilution of a concentrated liquid detergent, Aquet (Emil Greiner Company), to which is added three drops of 30% $H_2SO_4$ and enough acid fuchsin to give a bright red color. Acidification insures that only negligible amounts of carbon dioxide can be taken up by the manometer fluid. In the plastic cover of the animal chamber is a hole, plugged with a vaccine bottle stopper (9) and situated directly over a round, shallow plastic KOH dish (10), which is suspended by a screw from the plastic cover. To facilitate quantitative removal of the alkali, the dish is slightly funnel-shaped, has a small central depression, and is coated with a thin film of paraffin. The two arms of the manometer are open to air. When the instrument is in use, one opening is closed by a solid Lucite plug (11) and the other opening, in the arm leading to the animal chamber, is connected by a hollow Lucite taper (12) and eighth-inch plastic tubing (13) to a greased 10-milliliter hypodermic syringe (14), wired firmly to the animal chamber cover so that when the instrument is in the constant temperature bath, the syringe is under water. This syringe serves as a calibrated oxygen reservoir.

Figure 1B is a diagram of the gas analyzer used in the determination of the amount of carbon dioxide given off by a crab. To a Scholander plastic manometer

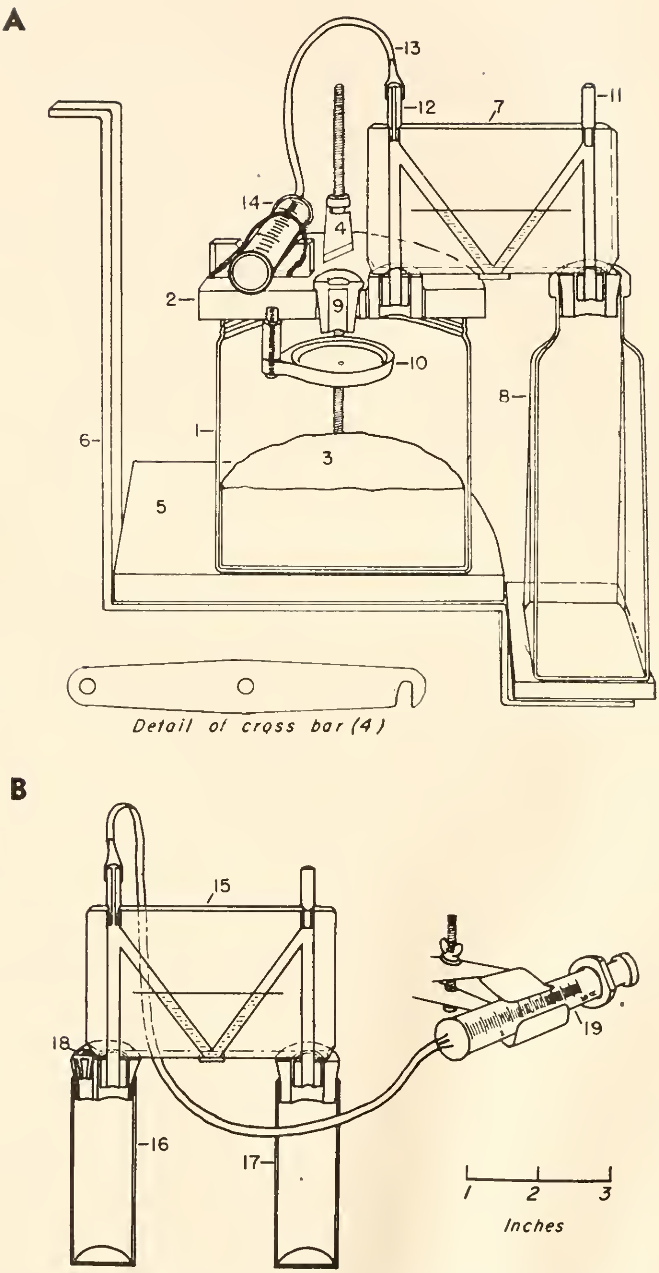

A
13
12
7
11
14
4
2
9
10
8
1
6
3
5
Detail of cross bar (4)
B
15
18
16
17
19
1
2
3
Inches

FIGURE 1. (A) Volumetric respirometer and (B) gas analyzer for volumetric determination of carbon dioxide, both drawn as if sectioned longitudinally through the midline. For explanation of figure numbers, see text.

(15) are attached two 18-milliliter glass shell vials, a dry vial (16) to act as a reaction vessel and a moistened one (17) to serve as a thermobarometer. In the rubber stopper of the reaction vessel is a small vaccine bottle stopper (18) and from the reaction vessel an arm of the manometer leads by means of plastic tubing to a 10- or 20-milliliter hypodermic syringe (19) which acts as the calibrated carbon dioxide measuring device. The manometer and shell vials are clamped in a constant temperature water bath and the carbon dioxide syringe is secured alongside the tank.

Procedure was standardized as follows: A crab was placed in the animal chamber, the instrument was assembled and clamped in the water bath, and the animal chamber was connected to the oxygen reservoir. Two milliliters of 10% KOH were injected into the shallow plastic dish through the vaccine bottle stopper just above it. After an equilibration period of 1½ to 2 hours, the KOH was removed by a hypodermic syringe, the needle point of which had been ground to a flat surface. The KOH was measured and discarded, the dish was rinsed with two milliliters of distilled water which was then quantitatively removed, and two milliliters of 10% KOH, which had been equilibrated to bath temperature (25.1° C.), were added. Immediately the first reading on the oxygen syringe was recorded. Readings to the nearest 0.05 of a milliliter were made at convenient intervals of from 10 to 45 minutes for a total time of 2½ to 4 hours. Most runs lasted about three hours and all runs were arranged to come in the afternoon, so that diurnal variations could be minimized. At the end of the run the KOH was quantitatively removed by a hypodermic syringe and injected at once into the reaction vessel of the gas analyzer. Eight-tenths of a milliliter of 30% $H_2SO_4$ was injected, the instrument was shaken vigorously until deflection of the manometer fluid reached a maximum (about three minutes), and the evolved carbon dioxide was measured on the attached syringe. This value was corrected for the carbon dioxide remaining in solution and for the carbonate originally present in the base. The crab was then removed from the animal chamber and weighed to the nearest tenth of a gram.

A significant portion of the carbon dioxide released from the KOH by acid remains in solution. Empirical data yielding the necessary correction

$$\left(\frac{\text{theoretical } CO_2 - \text{observed } CO_2}{\text{theoretical } CO_2}\right)$$

were obtained by the release within the gas analyzer of different volumes of carbon dioxide from standard carbonate solutions prepared so as to duplicate the carbonate-hydroxide mixtures resulting from an actual volumetric run. These standards were made by adding varying amounts (0.1–1.2 ml.) of 1.786 N $K_2CO_3$ to sufficient 1.786 N KOH (10% KOH) to give a total fluid volume of 2.0 ml. Gas release was accomplished by the addition of 0.8 ml. of 30% $H_2SO_4$ by weight (preparation: 20 ml. of concentrated $H_2SO_4$, 96% pure, plus 80 ml. of distilled water). Forty-eight determinations gave data in per cent which approximate the curve drawn in Figure 2. This curve was actually plotted from theoretical amounts of carbon dioxide calculated from the following formula:

$$V_t = V_o + \frac{V_o \propto V_f}{V_o + V_s},$$

where $V_t$ represents total $CO_2$, $V_o$ is observed $CO_2$, $V_f$ is the volume of fluid (2.8 ml.), $V_s$ is the gas capacity of the shell vial (14.2), and $\alpha$ represents the solubility coefficient for $CO_2$ at 25 degrees C. (0.570). This solubility coefficient was determined from available data (Seidell, 1940) for potassium acid sulfate and sulfuric acid of the same ionic strength as the solution resulting from the reaction between the acid and $KOH–K_2CO_3$. The solution was calculated to be approximately 3 N for both $H_2SO_4$ and $KHSO_4$. Although this equation was formulated independently, it is identical, when simplified, with that developed by Scholander, Claff, Andrews and Wallach (1952).

These theoretical and empirical results indicate that, under the stated experimental conditions, the correction which must be applied to an observed volume of carbon dioxide is almost 9% at a volume of 2.5 ml. but decreases to 4% at 24.0 ml. (Fig. 2). It should be emphasized that the position of the curve and therefore the per cent correction vary with temperature, concentration and amount of $KOH–K_2CO_3$, concentration and amount of $H_2SO_4$, and gas capacity of the reaction chamber. The carbon dioxide values from which the respiratory quotients presented in this paper were calculated have been corrected for the amount of carbon dioxide remaining in solution. Consequently, respiratory quotients are somewhat higher than those reported previously (Bliss, 1951, 1952) from the same respiratory data, since at the time of writing of those papers the error had not been recognized and determined.

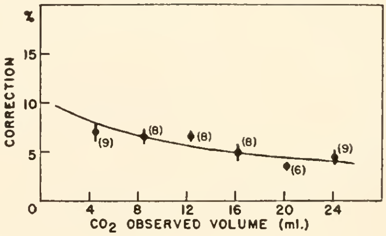


FIGURE 2. Theoretical curve and empirical data showing the correction in per cent which, due to the solubility of carbon dioxide in salt solutions, must be applied to observed volumes when carbon dioxide is evolved under the experimental conditions described in the text. Arithmetic means are indicated by closed circles and standard errors by vertical lines through these circles. Number of determinations indicated in parentheses.

Blank volumetric runs, performed under conditions identical with those of an experimental respiratory run but without the crab in the animal chamber, revealed that about 1½ hours were required for the respirometer to become equilibrated to the temperature of the water bath. After equilibration had been completed, blank runs of 8 hours' duration produced no measurable deflections of the manometer fluid. As indicated earlier, experimental procedure was standardized to include 1½ to 2 hours of equilibration.

This prolonged period of temperature equilibration served to insure chemical equilibration of the crab's body fluids with the carbon dioxide in transit between the animal and the KOH. A modification of the method described by Scholander, Claff, Andrews and Wallach (1952) was employed in determination of the amount of carbon dioxide in transit at any given carbon dioxide output rate. Carbon dioxide was released by 0.8 ml. of 30% $H_2SO_4$ from 2 ml. of $KOH–K_2CO_3$ in a Stender dish situated within the animal chamber of the respirometer. The volume of evolved gas, about 9.5 ml., was noted on the ten-milliliter greased syringe used for injection of the acid, the base of the syringe needle having, prior to the injection, been sealed into Tygon tubing and pushed firmly into the manometer port leading to the animal chamber. Two milliliters of 10% KOH were injected into the KOH dish. The carbon dioxide uptake by this KOH was followed on the syringe and plotted as a function of time. Tangents drawn to the carbon dioxide uptake curve, as described by Scholander, Claff, Andrews and Wallach (1952), indicated the amount of carbon dioxide in transit. This gas in transit, reaching concentrations within the animal chamber as high as 0.2% of the total gas volume during the respiration of a normal crab and up to 0.7% for a molting eyestalkless crab, can lower the total observed carbon dioxide and secondarily the respiratory quotient (1) by being untrapped in the alkali, and (2) by causing some carbon dioxide to be retained within the buffered body fluids of the crab. For these reasons, in the method described here, the KOH of the equilibration period does not contain the total amount of carbon dioxide produced by the crab during this period. However, this alkali is discarded. The KOH of the experimental run is added after the carbon dioxide of the crab's body fluids, the carbon dioxide in transit, and the carbon dioxide being trapped by the alkali have reached a steady state. Therefore all carbon dioxide produced by a crab during an experimental run is trapped in the KOH added at the beginning of the run. The recorded amount of carbon dioxide evolved from this KOH, when corrected for the gas remaining in solution, is a true measure of total carbon dioxide production during the experiment.

In order that the carbon dioxide buffer capacity of a crab might be estimated, the total oxygen consumption of two normal crabs was recorded first in the presence of KOH and immediately thereafter in its absence. The magnitude of the difference between these amounts, less than expected on the basis of an average respiratory quotient of 0.7 for these crabs as determined by the gas analysis method, was an indication of the power of a crab's body fluids to hold carbon dioxide in chemical combination. Clearly essential to the procurement of valid respiratory data is one of the following: (1) a low tension of carbon dioxide in transit; (2) an extended period of experimental observation and measurement; or (3), as in the method described here, a renewal of the carbon dioxide-absorbing KOH after a prolonged period of equilibration.

During experimental runs either with or without KOH, no sudden deflections of the manometer fluid, indicative of "bursts" of carbon dioxide, were observed. Such "bursts" have been described for insects by Punt (1950) and by H. A. Schneiderman and C. M. Williams (personal communication). The carbon dioxide output of a land crab at 25 degrees C. is steady and continuous. Carbon dioxide output by a land crab, at least at this temperature, can be taken as a true measure of its carbon dioxide production. If the gas is properly trapped by alkali and subsequently released from the alkali and measured, and if the measurement is corrected for solubility error, a valid respiratory quotient can be calculated.

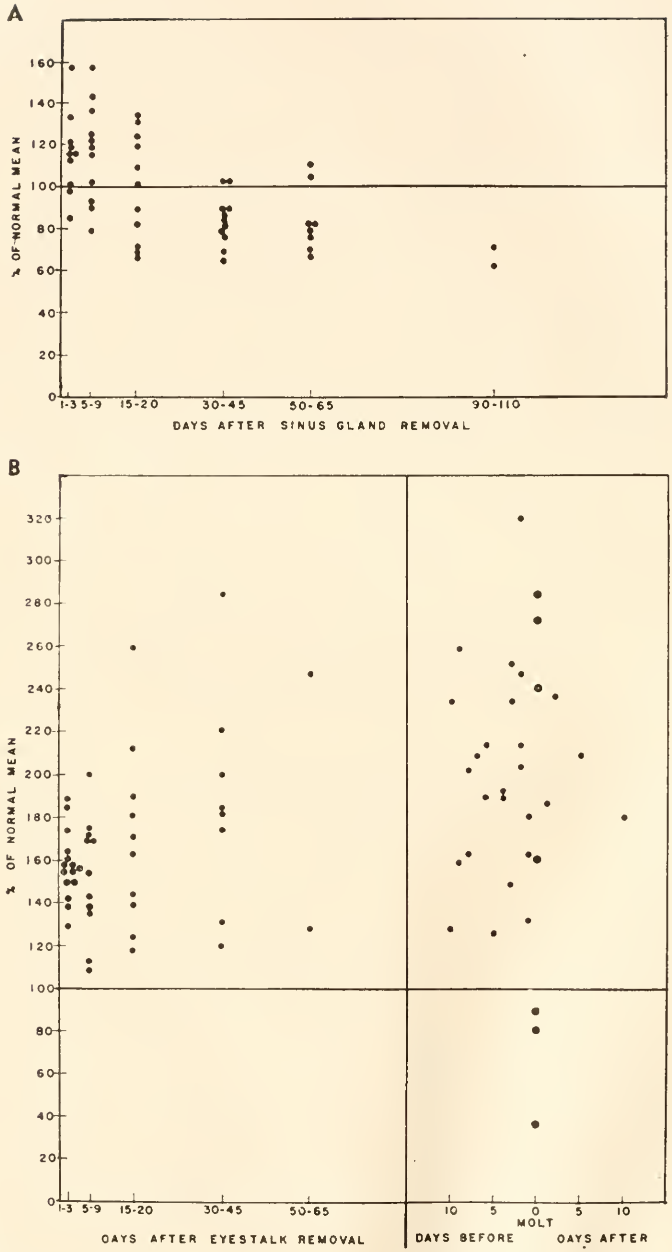


FIGURE 3. Rates of oxygen consumption of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. Each value is plotted as per cent of the normal mean for that animal.

## OBSERVATIONS

### *Contrasts in Metabolism Between Sinusglandless and Eyestalkless Crabs*

1. *Respiratory rate*

Rate of oxygen consumption was determined as mm.$^3$ per gram live weight per hour under standard conditions. Since it has been shown for the fiddler crab by Edwards (1950) that the absolute rate varies with sex and size, runs were limited to male animals, and all graphs intended to compare rates of many different animals have been expressed in per cent of each animal's normal average, as determined in two to four control runs.

Figures 3A and 3B indicate the striking differences between the rates of oxygen consumption of crabs from which only sinus glands have been removed and the respiratory rates of animals from which entire eyestalks have been taken. Figure 3A shows that after bilateral sinus gland removal, the rates of oxygen consumption tend in some animals to be high for the first few days but to be normal again within ten days to two weeks. The respiratory rates continue to drop, so that by the end of the first month they are below normal.

Bilateral eyestalk removal, on the other hand, causes the rates of oxygen consumption of all crabs to rise abruptly so that by the next day they are from 130% to 190% of normal. This rise may be followed by a temporary minor drop and then there is a gradual increase up to molt. On the day of molting, the respiratory rates have been variously recorded as 284% down to 36% of normal, but it is significant that the rates of those crabs which survived their molt were 284%, 273% and 240% of normal, whereas rates falling in the lower percentage range were recorded on animals which died during molt. It may be concluded that the respiratory rates are maximal at the time of molt. These observations on eyestalkless land crabs agree with those of Scudamore (1947) on crayfish and of Edwards (1950) on fiddler crabs. Scudamore, using the technique of Brown (1942), found that removal of sinus glands from three crayfish caused an increase in rates of oxygen consumption from a quarter to a fifth as great as the increase following eyestalk removal. Since his observations were limited to the first four days after surgery, he was probably observing in *Cambarus* a rise in respiratory rate comparable to that which the present writer has found may or may not occur in *Gecarcinus* following sinus gland removal and which, in any case, has disappeared within the ten days to two weeks following the operation.

2. *Respiratory quotient*

Each respiratory quotient was calculated as the total amount of carbon dioxide produced divided by the total amount of oxygen consumed during an experimental run.

The respiratory quotients of the entire series of normal animals tested from the beginning of November, 1950, until the end of June, 1951, are plotted in Figure 4. Each circle represents an individual determination and two to four separate circles represent the respiratory quotients for one animal. Therefore the one hundred and forty-odd circles represent the respiratory quotients of about forty animals. There is considerable variation in the normal respiratory quotient, which may range as high as 0.97 or as low as 0.61. The standard deviation of

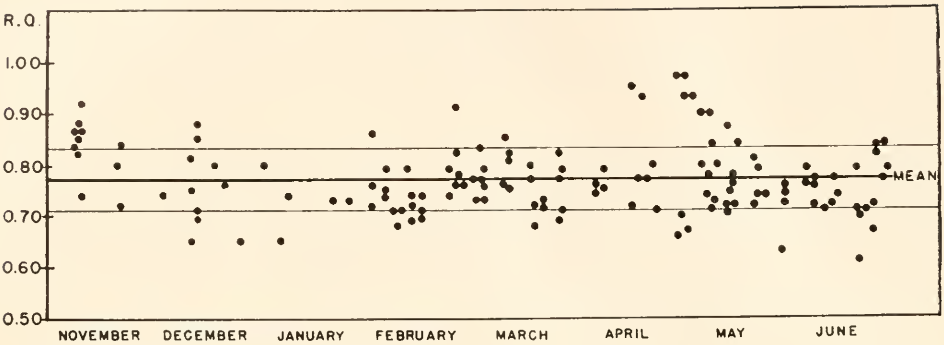


FIGURE 4. Respiratory quotients recorded from normal *Gecarcinus lateralis* from November, 1950, through June, 1951. The heavy line represents the mean respiratory quotient and the two narrow lines on either side of the mean indicate the range of plus and minus one standard deviation.

the mean respiratory quotients of 37 crabs is ±0.06. The mean value is 0.77 ±0.01.

In Figure 5A are shown the respiratory quotients for crabs from which both sinus glands have been removed. Each circle on the graph represents an individual determination but in this case the values are grouped, so that all determinations made between the first and the third day after sinus gland removal are in one column, those made between the fifth and ninth days in another, and so on. These values have an essentially normal distribution although there is a tendency, probably not of statistical significance (Table I), for a drop in respiratory quotient

TABLE I

*The respiratory quotients of Gecarcinus lateralis*

| | Number in sample | Arithmetic mean and standard error | Probability (*P*)* |
|---|---|---|---|
| Normal Crabs | 37 | 0.77 ± 0.01 | |
| Eyestalkless Crabs | | | |
| 1–3 days after operation | 7 | 0.63 ± 0.02 | <0.001 |
| 5–9 days after operation | 6 | 0.69 ± 0.01 | 0.001 |
| 7–20 days pre-molt | 16 | 0.68 ± 0.01 | <0.001 |
| Molting | 7 | 1.40 ± 0.14 | <0.001 |
| Sinusglandless Crabs | | | |
| 1–3 days after operation | 10 | 0.72 ± 0.02 | 0.02 |
| 5–9 days after operation | 11 | 0.74 ± 0.02 | 0.13 |
| 15–20 days after operation | 11 | 0.79 ± 0.02 | 0.43 |
| 30–45 days after operation | 11 | 0.77 ± 0.02 | 0.93 |
| 50–65 days after operation | 8 | 0.75 ± 0.03 | 0.28 |

* *P* values are from table of *t* as given in Fisher and Yates (1948). A difference in mean respiratory quotient between normal and operated crabs is considered highly significant if *P* is less than 0.01.

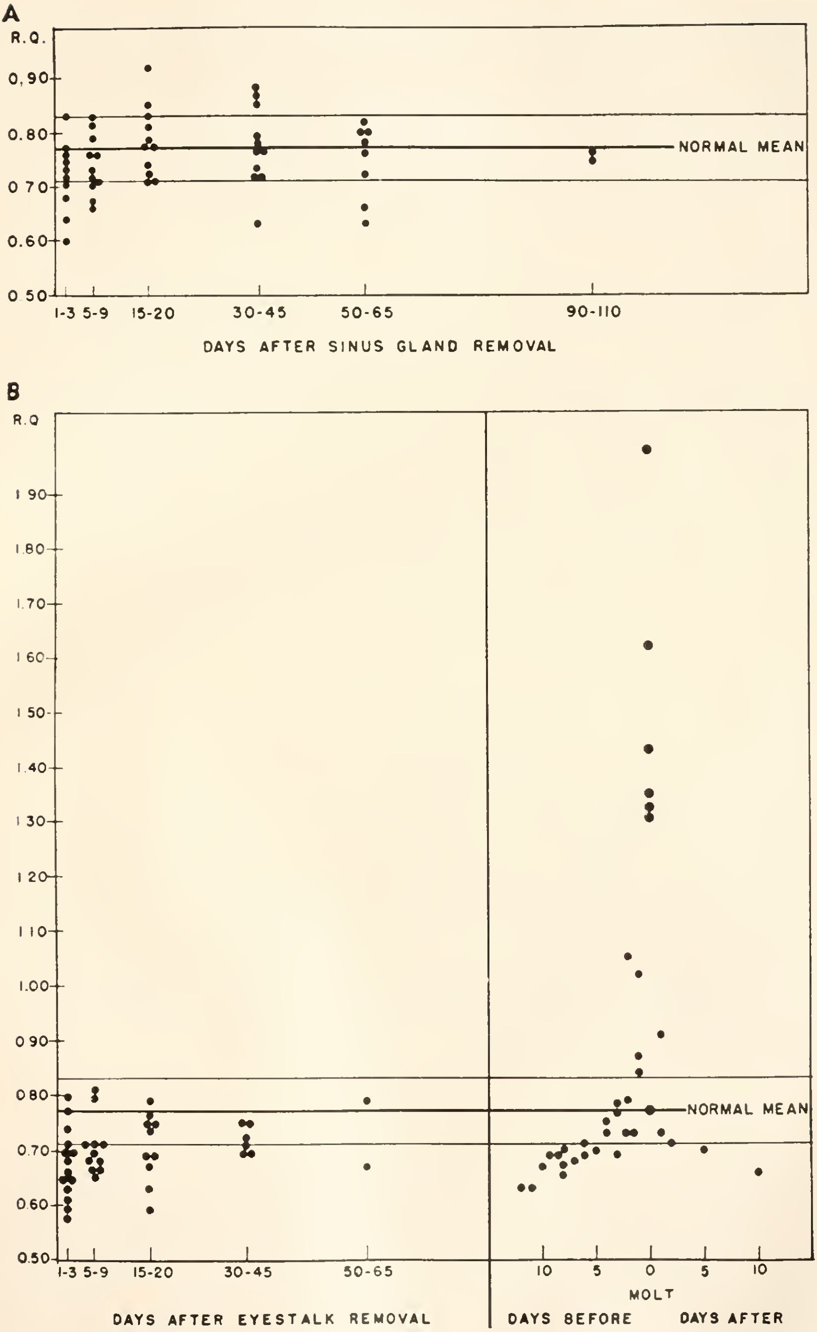


FIGURE 5. Respiratory quotients of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. The normal mean and standard deviation are indicated as in Figure 4.

during the first few days after the operation, then a recovery to or above normal, and finally a leveling at approximately the normal mean value.

Removal of both eyestalks causes a radical change in respiratory quotient (Fig. 5B). Three days after operations performed in the spring months, the mean respiratory quotient has dropped to 0.63 ± 0.02 (Table I). By the fifth day the mean has risen somewhat to 0.69 ± 0.01, remaining near this value until around the tenth day before molt. A gradual rise, which starts at this time, culminates in a precipitous increase on the day of molt, when values as high as 1.97 have been recorded. Subsequently the mean respiratory quotient falls to the pre-molt level.

The rapidity and magnitude of response to eyestalk removal depends upon the time of year at which the operation is performed. Operations on four crabs during January and February produced no clear effect on respiratory quotients. Three weeks after the operations, the respiratory quotients of these crabs dropped to values which, although subnormal, were generally higher than those recorded from crabs made eyestalkless during the spring. The January-February data are responsible for the circles in Figure 5B (left side) which lie close to the line representing the normal mean. Similarly rates of oxygen consumption from these crabs are indicated by the circles in Figure 3B nearest to the 100% line. Since respiratory quotients recorded in January and February were from crabs which were not immediately responsive to eyestalk removal, these data have been omitted from calculations for respiratory quotients typical of crabs 1–3 and 5–9 days after eyestalk removal (Table I). It is interesting to note, however, that when data from non-responsive eyestalkless crabs of the winter months are included in the calculations, the mean respiratory quotients of 0.67 ± 0.02 for 1–3 days and 0.71 ± 0.01 for 5–9 days are still significantly below the normal mean of 0.77 ± 0.01, with P values of less than 0.001 and 0.01, respectively.

Intervals between eyestalk removal and molt become shorter with the advance of the seasons. In January and February forty to fifty days intervene between operation and molt, whereas in June only twenty to thirty days elapse. One may postulate that in the spring, eyestalk removal throws a crab at once into a full-fledged growth and molt metabolism, with an accompanying drop in respiratory quotient and rise in respiratory rate. During the winter months this shift in metabolism is partially blocked, so that the growth processes which are triggered by eyestalk removal start more slowly, proceed at reduced rates, and require longer periods of time for their completion.

### 3. *Water uptake and body weight*

Increases in water uptake and weight were first reported by Scudamore (1947) for eyestalkless crayfish and for crayfish from which sinus glands had been removed. Figure 6A shows that removal of both sinus glands from *Gecarcinus lateralis* did not cause the weight of the crabs to deviate significantly from normal. Increase in weight followed eyestalk removal, the greater portion of the increase commencing about 10 days before molt, with maximum values on the day of molt (Fig. 6B).

Responsible for the pre-molt weight increase in eyestalkless crabs was their water uptake, becoming so marked as molt approached that the membrane linking abdomen to thorax bulged as if about to burst. The weights recorded on sinus-glandless crabs remained approximately unchanged because no increase in water uptake occurred. Intersegmental membranes were normal in appearance.

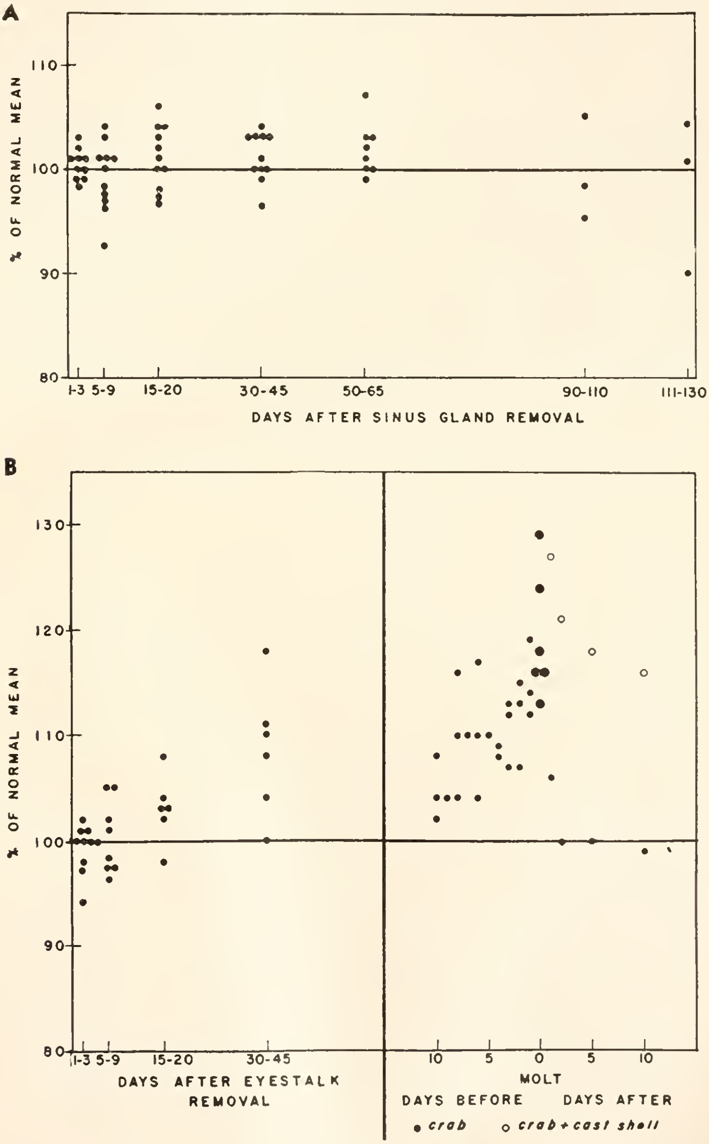


FIGURE 6. Wet weights of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. Each value is plotted as per cent of the normal mean for that animal.

### 4. *Molt and gastrolith formation*

From the observations summarized in the preceding paragraphs it is apparent that, along with the relatively normal respiratory rate, respiratory quotient and water uptake which characterize sinusglandless crabs, there is no induction of molt. Eyestalk removal causes molt, with its preliminary alterations in respiratory metabolism and water balance, to occur. During the period of experimentation and observation (November, 1950, through June, 1951) only one normal crab molted.

Coincident with other preparations for molt, eyestalkless crabs remove calcium from the exoskeleton and deposit it as gastroliths under the chitinous lining of the stomach. Although from about the tenth day after eyestalk removal four pearly-white gastroliths form upon the calcium carbonate framework of the stomach, they do not appear after sinus gland removal. Scudamore (1947, p. 192) reported that in crayfish "bilateral sinus-gland extirpation resulted in gastrolith formation in all cases, although at a slightly slower rate than after bilateral eyestalk ablation." His data, however, reveal that 17 days after surgery the gastroliths of sinusglandless crayfish weighed only 23% as much as did those of eyestalkless animals.

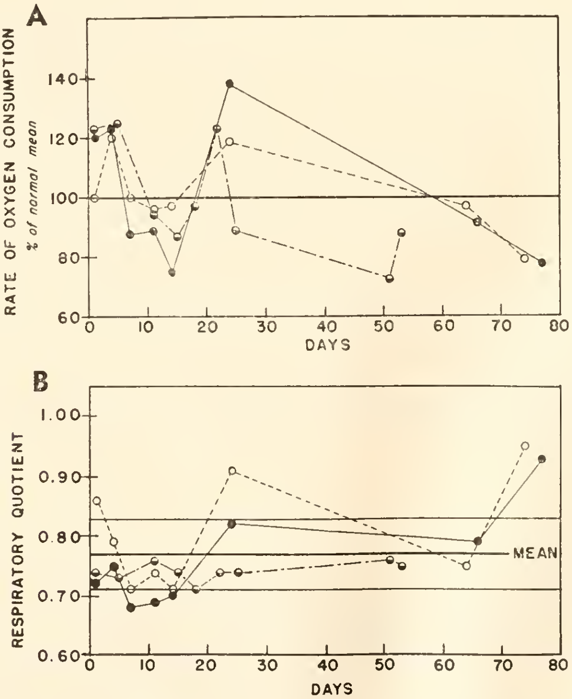


FIGURE 7. (A) Rates of oxygen consumption and (B) respiratory quotients of three normal crabs recorded over periods up to 77 days. The respiratory rates are indicated as per cent of each animal's mean. The normal mean respiratory quotient and standard deviation are indicated as in Figure 4.

## *The Respiratory Metabolism of Individual Crabs*

The general pattern of response that is visible in group data presented in the previous section is apparent when the respiratory metabolism of individual crabs is followed for long periods.

### 1. *Normal respiratory metabolism*

In Figure 7A are plotted the rates of oxygen consumption of three crabs over a period of 77 days. Figure 7B shows the corresponding respiratory quotients during the same respiratory runs. Visible in both graphs is considerable fluctuation, indicating that a normal crab varies both its rate and type of metabolism. From a mean respiratory quotient of 0.77, it can be tentatively assumed that the foods principally oxidized are proteins and fats. Oil globules are visible in great quantities in the hepatopancreas. Fluctuations of respiratory quotient suggest, however, that conversion of carbohydrate to fat, producing higher respiratory quotients, and conversion of fat to carbohydrate, yielding lower values, may be involved in normal maintenance metabolism. The lower, steadier respiratory quotients recorded after eyestalk removal suggest that the fluctuating metabolism of the normal crab has been replaced by a relatively invariable metabolism, in

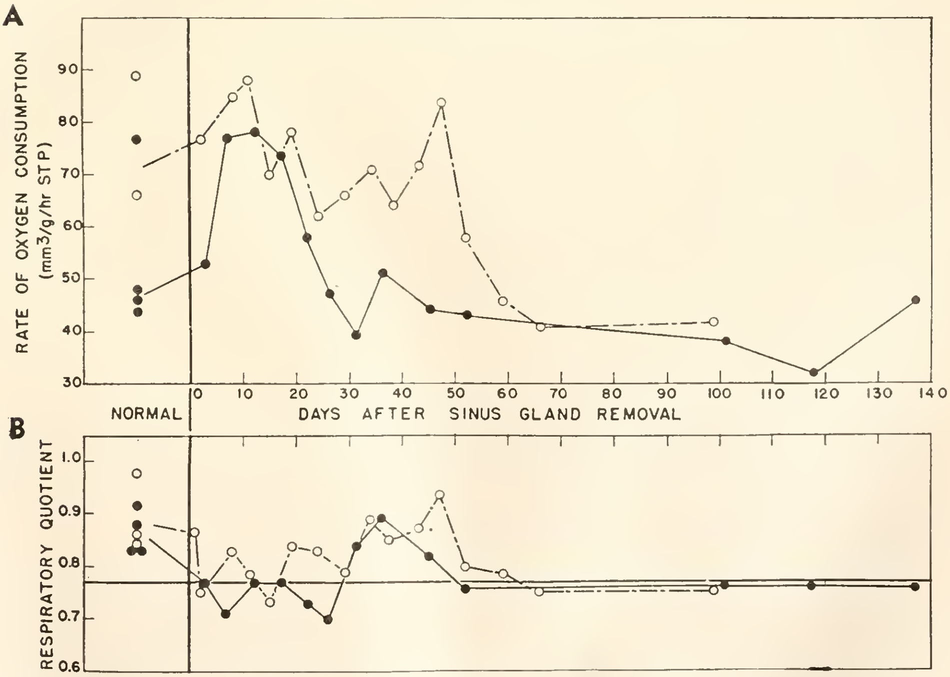


FIGURE 8. (A) Rates of oxygen consumption and (B) respiratory quotients of two crabs when normal and after bilateral removal of sinus glands. Open circles: crab 2; closed circles: crab 4. Solid horizontal line in (B): normal mean respiratory quotient.

which oxidation of fats and conversion of fats into organic acids, carbohydrates and other raw materials for the synthesis essential to growth play important roles.

### 2. *Respiratory metabolism after bilateral sinus gland removal*

One to two months after bilateral sinus gland removal there occurred stabilization of the respiratory quotient (Fig. 8B) close to the normal mean of 0.77 and of the respiratory rate (Fig. 8A) at normal or sub-normal levels. In six out of eight cases, of which one (crab 4) is shown in Figure 8A and another (crab 26) is illustrated in Figure 9C, the respiratory rate ascended immediately after the operation and then declined almost without fluctuations to reach, within the first month, a steady normal or sub-normal level. In the remaining two cases, of which one is illustrated in Figure 8A (crab 2), the respiratory rate subsequent to sinus gland removal varied near this crab's normal mean before stabilizing at a steady low level.

In four out of eight sinusglandless crabs, including crabs 2 and 4 of Figure 8B, the respiratory quotient fluctuated around the normal mean before stabilizing there, but in the remaining four individuals, of which one (crab 26) is shown in Figure 9D, the respiratory quotient fell sharply immediately after sinus gland removal and within three weeks had climbed smoothly to normal, where it stabilized.

In a previous paper (Bliss and Welsh, 1952) it was reported that a sinus gland starts to regenerate in an atypical position on the medulla terminalis of each eyestalk shortly after bilateral sinus gland removal. It was demonstrated that normal and regenerated "glands" are masses of swollen nerve endings containing secretory material which has been produced in neurosecretory cells of the central nervous system and transported along their axons to these reservoirs. By the time the respiratory metabolism has stabilized, the crab has substitute sinus glands. Yet regenerated glands of one or two months cannot be functioning like the original structures, since the respiratory metabolism is not fluctuating as it does in a normal animal. The long-term respiratory picture suggests that sooner or later a crab from which the original sinus glands have been removed is without the ability to vary the amount and the type of its metabolism.

### 3. *Respiratory metabolism after removal of one eyestalk*

Removal of one eyestalk may produce a slight rise in rate of oxygen consumption or, as in crab 21 (Fig. 9A), no change. A temporary drop in respiratory quotient may occur (Fig. 9B). Subsequent removal of the other eyestalk from the same crab causes a permanent pre-molt rise in respiratory rate and fall in respiratory quotient, as in other eyestalkless crabs (Figs. 3B, 5B).

Bauchau (1948b) obtained comparable results in *Eriocheir sinensis*, observing a 25% increase in respiratory rate five days after unilateral eyestalk removal and a sharp rise after removal of the second eyestalk. Edwards (1950) reported a respiratory rate 36% above normal when *Uca* from which one eyestalk had been removed were tested 14 to 39 days after the operation. The rate rose to 162% of normal when the second eyestalk was ablated.

The small rise observed by Bauchau after unilateral eyestalk removal was attributed by him to insufficiency of secretion from the remaining sinus gland. If this interpretation is correct, Edwards' results indicate that compensation for the shortage of hormone by release of gradually increasing amounts from the intact gland does not occur. The slight intensification of respiratory metabolism correlates with the slight acceleration of molt frequency observed by Brown and Cunningham (1939) in crayfish possessing only one eyestalk.

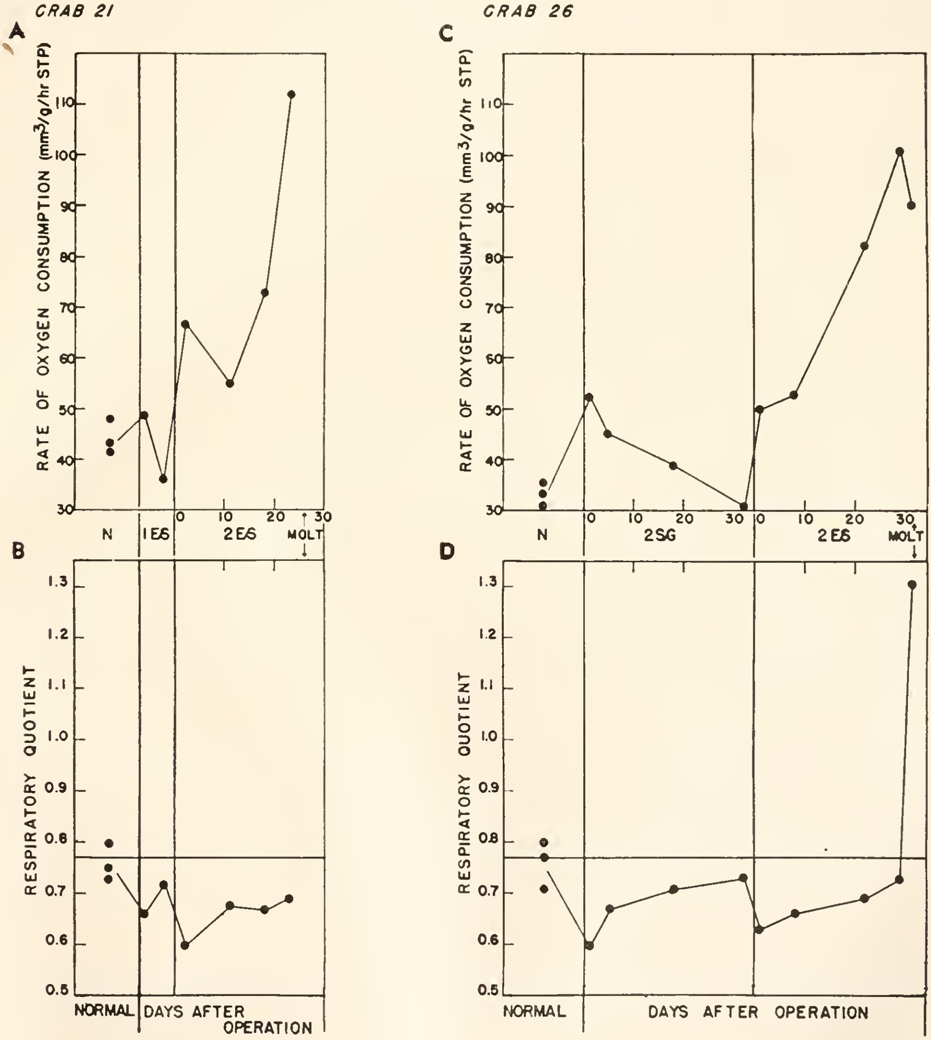


FIGURE 9. (A) Rates of oxygen consumption and (B) respiratory quotients of crab 21 when normal (N), after removal of one eyestalk (1ES) and after removal of the second eyestalk (2ES). The respiratory rates (C) and respiratory quotients (D) of crab 26 are illustrated when the crab is normal (N), without its sinus glands (2SG), and later without its eyestalks (2ES). Solid horizontal line in (B) and (D): normal mean respiratory quotient.

### 4. *Respiratory metabolism when bilateral sinus gland removal is followed by bilateral eyestalk removal*

The usual responses to eyestalk removal occur just as if there had been no previous surgical treatment, when bilateral eyestalk removal follows some time after bilateral sinus gland removal (Figs. 9C, 9D). These responses, recorded in three crabs, indicate that (1) the ability to respond to eyestalk removal is not altered by previous sinus gland removal, and (2) the molt-inhibitory hormone is permanently withdrawn or effectively reduced in concentration not by sinus gland removal but by eyestalk removal.

## Discussion

Marked differences between the effects on respiration of sinus gland removal and those of eyestalk removal, coupled with contrasts in water uptake, gastrolith formation and induction of molt, have led the writer inevitably to the conclusion that the removal of sinus glands in *Gecarcinus lateralis* is not equivalent to bilateral eyestalk removal. Observations by Passano (1951b, 1952b) on the incidence of molting in crabs after eyestalk removal and after sinus gland removal (technique modified from Kleinholz, 1947) are in complete harmony with the results reported in the present paper. Furthermore, Passano and the writer separately have found that the sinus gland of crabs is connected by a large nerve tract with the x-organ[3] (Passano, 1951a, 1951b, 1952b; Bliss, 1951, 1952). Passano (1951b, 1952b), in an extensive series of experiments has shown that x-organ removal, like eyestalk removal, can induce molt and that implants of x-organs into eyestalkless crabs can delay molting. Passano has demonstrated for the first time a function, molt inhibition, for the x-organ. Results of two experiments by the present writer are in agreement with this work of Passano. Bilateral removal of x-organ and x-organ nerve, in one case with and in the other case without simultaneous removal of sinus glands, brought the same respiratory and molting responses in *Gecarcinus lateralis* as did bilateral eyestalk removal. On the basis of physiological and morphological evidence it was suggested independently by Bliss and Passano that a hormone[4] is synthesized in the x-organ of crabs and transported by way of a nerve to the sinus gland. Subsequent studies by Bliss and Welsh (1952) have demonstrated morphologically that many groups of neurosecretory cells situated in ganglia throughout the central nervous system of crabs participate in the synthesis of secretory material and in its transport along their axons to the two storage-release centers, the sinus glands. These more recent observations can explain the effectiveness of certain eyestalk tissues other than x-organs in delaying molt when, as reported by Passano (1951b, 1952b), they are implanted into eyestalkless crabs.

The conclusions presented in this paper that processes triggered by bilateral eyestalk removal are not evoked by bilateral sinus gland removal contrast with conclusions published by Scudamore (1947). Scudamore wrote (p. 205), "Bilateral eyestalk or sinus-gland excision resulted in gastrolith formation, removal of soluble salts from the exoskeleton, increase in water content, increase in oxygen consumption, and molting. . . ." *Cambarus,* used by Scudamore, responded to sinus gland removal ( at 20% to 40% of the eyestalkless crayfish response with, however, only one molt) whereas, with the exception of a temporary change in mean respiratory rate and quotient, *Gecarcinus,* used by the present writer, did not respond in a molt-preparatory manner. In the light of recent physiological and morphological observations on crayfish, summarized in the next paragraph, it is probable that these contrasting results may be explained in terms of differing techniques of sinus gland removal. Proximity of the sinus gland to many neurosecretory centers within the eyestalk (see Bliss and Welsh, 1952, Figs. 1–3) requires that destruction of eyestalk tissue either during or following an operation be minimized. The method of sinus gland removal described in the present paper has the advantage, in common with the technique devised by Kleinholz (1947), of facilitating removal of a sinus gland with little derangement of other eyestalk tissues.

---

[3] Probably the same discovery as was made in *Sesarma* by Enami (1951) who described these neurosecretory cells as "beta cells."

[4] Other than to suggest that the molt-inhibiting and respiration-regulating principles may be identical, the present studies have led to no conclusions concerning the number, identity and interrelationships of hormones being released by the sinus gland.

There are now indications that in crayfish the sinus glands can be removed without permanent interference with metabolism. Normal blood calcium levels were maintained and no incidence of molting occurred in crayfish from which sinus glands had been removed (Havel, 1949; Havel and Kleinholz, 1951). Sinus glandless crayfish were found to have a rate of oxygen consumption which was only slightly higher than that recorded after mock sinus gland operation and considerably below that of eyestalkless animals (Frost, 1948; Saloum, 1951; Frost, Saloum and Kleinholz, 1951). Mr. James B. Durand, Jr. (personal communication) has observed that nerve fibers from the x-organ and brain of crayfish carry stainable secretory material to their swollen endings, the sinus glands. In Macrura as in Brachyura there appears to be a neurosecretory system in which the sinus glands play the role of storage-release centers. The concept of a neurosecretory system, developed originally for vertebrates and insects (Scharrer and Scharrer, 1944; Bargmann and Scharrer, 1951; Scharrer, 1951; Scharrer, 1952a, 1952b) has now been extended to include the decapod Crustacea (Bliss and Welsh, 1952).

The writer wishes to express sincere thanks to Professor John H. Welsh for his constant encouragement, criticism and advice throughout the course of this study. Grateful acknowledgment is made to Dr. P. F. Scholander, Dr. Howard A. Schneiderman, Dr. Conrad Yocum and Dr. Richard Diamond for invaluable advice concerning respirometer practice and theory.

## SUMMARY

1. A volumetric macrorespirometer and gas analyzer are described. Procedures and precautions for their use are discussed.

2. Determinations of respiratory rates and respiratory quotients before and after surgical removal of both sinus glands from the eyestalks of the land crab, *Gecarcinus lateralis,* indicated that, except in one respect, the crabs were fundamentally unaffected by the loss of organs which had been thought essential for maintenance of normal metabolism.

3. The exception lay in an operated crab's eventual loss of its normal ability to vary the type and rate of metabolism. Indications of this loss were a constant respiratory quotient at the normal mean value and a low and relatively invariable respiratory rate. Fluctuations in rate of oxygen consumption and level of respiratory quotient were recorded from normal crabs.

4. Eyestalkless crabs showed a sudden and pronounced alteration from normal respiratory rates and respiratory quotients, a change indicative of a pre-molt metabolism and culminating in a second, even more marked metabolic shift at the time of molt.

5. It is clear that removal of eyestalks does, but removal of sinus glands does not, deprive these crabs of the molt-inhibiting and respiration-regulating hormone. Previous bilateral sinus gland removal does not alter the capacity of a crab to respond to subsequent bilateral eyestalk removal in the manner described above. The same is true when unilateral eyestalk removal is followed by removal of the other eyestalk.

6. These metabolic data supplement and confirm morphological observations reported in an earlier paper (Bliss and Welsh, 1952). One may conceive of a brachyuran neurosecretory system composed of neurosecretory cell bodies as synthesizing elements, axons as transporting elements, and the sinus glands as storing and releasing organs. This is an extension of a concept developed originally by Scharrer and Scharrer (1944) for vertebrates and insects.

## LITERATURE CITED

Abramowitz, A. A., and R. K. Abramowitz, 1938. On the specificity and related properties of the crustacean chromatophorotropic hormone. *Biol. Bull.,* **74**: 278–296.

Abramowitz, R. K., and A. A. Abramowitz, 1940. Moulting, growth, and survival after eyestalk removal in *Uca pugilator. Biol. Bull.,* **78**: 179–188.

Bargmann, W., and E. Scharrer, 1951. The site of origin of the hormones of the posterior pituitary. *Amer. Sci.,* **39**: 255–259.

Bauchau, A. G., 1948a. Phénomènes de croissance et glande sinusaire chez *Eriocheir sinensis.* H. M. Edw. *Ann. Soc. Roy. de Zool. de Belg.,* **79**: 125–131.

Bauchau, A. G., 1948b. Intensité du métabolisme et glande sinusaire chez *Eriocheir sinensis.* H. M. Edw. *Ann Soc. Roy. de Zool. de Belg.,* **79**: 73–86.

Bliss, Dorothy E., 1951. Metabolic effects of sinus gland or eyestalk removal in the land crab, *Gecarcinus lateralis. Anat. Rec.,* **111**: 502.

Bliss, Dorothy E., 1952. Endocrine control of metabolism in the decapod crustacean, *Gecarcinus lateralis.* Thesis for Ph.D. degree, Radcliffe College.

Bliss, Dorothy E., and J. H. Welsh, 1952. The neurosecretory system of brachyuran Crustacea. *Biol. Bull.,* **103**: 157–169.

Brown, F. A., Jr., 1935. Control of pigment migration within the chromatophores of *Palaemonetes vulgaris. J. Exp. Zool.,* **71**: 1–15.

Brown, F. A., Jr., 1940. The crustacean sinus gland and chromatophore activation. *Physiol. Zool.,* **13**: 343–355.

Brown, F. A., Jr., 1942. Sinus gland extirpation in the crayfish without eyestalk removal. *Proc. Soc. Exp. Biol. Med.,* **50**: 295–297.

Brown, F. A., Jr., and Ona Cunningham, 1939. Influence of the sinus gland of crustaceans on normal viability and ecdysis. *Biol. Bull.,* **77**: 104–114.

Edwards, G. A., 1950. The influence of eyestalk removal on the metabolism of the fiddler crab. *Physiol. Comp. Oecol.,* **2**: 34–50.

Enami, M., 1951. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma.* II. Histology of incretory elements. *Biol. Bull.,* **101**: 241–258.

Fisher, R. A., and F. Yates, 1948. Statistical tables for biological, agricultural and medical research. 3rd ed. Edinburgh.

Flemister, L. J., and Sarah C. Flemister, 1951. Chloride ion regulation and oxygen consumption in the crab *Ocypode albicans* (Bosq). *Biol. Bull.*, **101**: 259–273.

Frost, R. C., 1948. The role of the sinus gland in relation to oxygen consumption in the crayfish. Thesis for A.B. degree, Reed College.

Frost, R., R. Saloum and L. H. Kleinholz, 1951. Effect of sinus gland and of eyestalk removal on rate of oxygen consumption in *Astacus*. *Anat. Rec.*, **111**: 572.

Hanström, B., 1931. Neue Untersuchungen über Sinnesorgane und Nervensystem der Crustaceen. I. *Morphol. u. Ökol. d. Tiere*, 23: 80–236.

Hanström, B., 1933. Neue Untersuchungen über Sinnesorgane und Nervensystem der Crustaceen. II. *Zool. Jahrb. Abt. Anat. Ont. Tiere*, **56**: 387–520.

Hanström, B., 1939. Hormones in invertebrates. Oxford Univ. Press.

Havel, Virginia J., 1949. Some aspects of calcium metabolism in relation to molting in Crustacea. Thesis for A.M. degree, Reed College.

Havel, Virginia J., and L. H. Kleinholz, 1951. Effect of seasonal variation, sinus gland removal, and eyestalk removal on concentration of blood calcium in *Astacus*. *Anat. Rec.*, **111**: 571.

Kleinholz, L. H., 1934. Eye-stalk hormone and the movement of distal retinal pigment in *Palaemonetes*. *Proc. Nat. Acad. Sci.*, **20**: 659–661.

Kleinholz, L. H., 1936. Crustacean eye-stalk hormone and retinal pigment migration. *Biol. Bull.*, **70**: 159–184.

Kleinholz, L. H., 1947. A method for removal of the sinus gland from the eyestalks of crustaceans. *Biol. Bull.*, **93**: 52–55.

Kleinholz, L. H., 1948. Factors controlling the migration of the proximal pigment of the crustacean retina. *Anat. Rec.*, **101**: 665.

Kleinholz, L. H., 1949a. Responses of the proximal retinal pigment of the isolated crustacean eyestalk to light and to darkness. *Proc. Nat. Acad. Sci.*, **35**: 215–218.

Kleinholz, L. H., 1949b. Migrations of the retinal pigments and their regulation by the sinus gland. *Bull. Biol. de France et de Belg., Suppl.*, **33**: 127–138.

Kleinholz, L. H., and Emma Bourquin, 1941. Effects of eyestalk removal on decapod crustaceans. *Proc. Nat. Acad. Sci.*, **27**: 145–149.

Koller, G., 1925. Farbwechsel bei *Crangon vulgaris*. *Verh. deutsch. zool. Gesells.*, **30**: 128–132.

Koller, G., 1927. Über Chromatophorensystem, Farbensinn und Farbwechsel bei *Crangon vulgaris*. *Zeitschr. vergl. Physiol.*, **5**: 191–246.

Kyer, D. L., 1942. The influence of the sinus glands on gastrolith formation in the crayfish. *Biol. Bull.*, **82**: 68–78.

Megušar, F., 1912. Experimente über den Farbwechsel der Crustacean. *Arch. f. Entw.*, **33**: 462–665.

Passano, L. M., 1951a. The X organ-sinus gland neurosecretory system in crabs. *Anat. Rec.*, **111**: 502.

Passano, L. M., 1951b. The X organ, a neurosecretory gland controlling molting crabs. *Anat. Rec.*, **111**: 559.

Passano, L. M., 1952a. Phase contrast observations on living neurosecretory cells of *Sesarma*. *Anat. Rec.*, **112**: 460–461.

Passano, L. M., 1952b. The X organ-sinus gland complex of brachyuran crustaceans, a neurosecretory molt controlling gland. Thesis for the Ph.D. degree, Yale University.

Perkins, E. B., 1928. Color changes in crustaceans, especially in *Palaemonetes*. *J. Exp. Zool.*, **50**: 71–105.

Punt, A., 1950. The respiration of insects. *Physiol. Comp. Oecol.*, 2: 59–74.

Saloum, R. D., 1951. The effect of the sinus gland on general metabolism as indicated by the rate of oxygen consumption in *Astacus*. Thesis for the A.B. degree, Reed College.

Scharrer, Berta, 1952a. Neurosecretion. XI. The effects of nerve section on the intercerebralis-cardiacum-allatum system of the insect *Leucophaea maderae*. *Biol. Bull.*, **102**: 261–272.

SCHARRER, BERTA, 1952b. Über neuroendokrine Vorgänge bei Insekten. *Pflügers Arch.*, **255**: 154–163.

SCHARRER, BERTA, AND E. SCHARRER, 1944. Neurosecretion. VI. A comparison between the intercerebralis-cardiacum-allatum system of the insects and the hypothalamo-hypophyseal system of the vertebrates. *Biol. Bull.*, **87**: 242–251.

SCHARRER, E., 1951. Neurosecretion. X. A relationship between the paraphysis and the paraventricular nucleus in the garter snake (*Thamnophis* sp.). *Biol. Bull.*, **101**: 106–113.

SCHOLANDER, P. F., 1949. Volumetric respirometer for aquatic animals. *Rev. Sci. Instr.*, **20**: 885–887.

SCHOLANDER, P. F., C. L. CLAFF, J. R. ANDREWS AND D. F. WALLACH, 1952. Microvolumetric respirometry. Methods for measuring $O_2$ consumption and $CO_2$ production by cells and enzymic reactions. *J. Gen. Physiol.*, **35**: 375–395.

SCUDAMORE, H. H., 1942. Influence of the sinus glands upon certain metabolic changes associated with molting in the crayfish, *Cambarus immunis*. *Anat. Rec.*, **84**: 515.

SCUDAMORE, H. H., 1947. The influence of sinus glands upon molting and associated changes in the crayfish. *Physiol. Zool.*, **20**: 187–208.

SEIDELL, A., 1940. Solubilities of inorganic and metal organic compounds. Vol. I. 3rd ed., Van Nostrand Co., New York, N. Y.

SMITH, R. I., 1940. Studies on the effects of eyestalk removal upon young crayfish (*Cambarus clarkii* Girard). *Biol. Bull.*, **79**: 145–152.

TRAVIS, DOROTHY F., 1951a. Calcium metabolism in the decapod Crustacea. Thesis for the Ph.D. degree, Radcliffe College.

TRAVIS, DOROTHY F., 1951b. The control of the sinus glands over certain aspects of calcium metabolism in *Panulirus argus* Latreille. *Anat. Rec.*, **111**: 503.

WELSH, J. H., 1930a. The mechanics of migration of the distal pigment cells in the eyes of *Palaemonetes*. *J. Exp. Zool.*, **56**: 459–494.

WELSH, J. H., 1930b. Diurnal rhythm of the distal pigment cells in the eyes of certain crustaceans. *Proc. Nat. Acad. Sci.*, **16**: 386–395.

WELSH, J. H., 1939. The action of eye-stalk extracts on retinal pigment migration in the crayfish, *Cambarus bartoni*. *Biol. Bull.*, **77**: 119–125.

WELSH, J. H., 1941. The sinus glands and 24-hour cycles of retinal pigment migration in the crayfish. *J. Exp. Zool.*, **86**: 35–49.

WELSH, J. H., 1951. New evidence concerning the source and action of the eyestalk hormone. *Anat. Rec.*, **111**: 442.

WILLIAMS, C. M., 1946. Continuous anesthesia for insects. *Science*, **103**: 57.

ZELENY, C., 1905. Compensatory regulation. *J. Exp. Zool.*, **2**: 1–102.

# VARIATIONS IN CELL SIZE DURING THE DEVELOPMENT OF THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM[1]

JOHN TYLER BONNER AND EVELYN BARBARA FRASCELLA

*Department of Biology, Princeton University, Princeton, New Jersey*

In many organisms, especially multicellular ones, it is relatively difficult to obtain an estimate of individual cell sizes, but in the amoeboid slime mold *Dictyostelium discoideum* it is not only possible to do this with reasonable accuracy for one stage, but for most of the stages of development. In the life cycle of this slime mold (Raper, 1935, 1951; Bonner, 1944) the amoebae are first separate during their actively feeding stage, and later after a short period of fasting they aggregate to form sausage-shaped cell masses which migrate for variable periods of time. During this migration stage the anterior cells begin their differentiation into stalk cells and the posterior cells begin their differentiation into spores (Bonner, 1952) and then finally the migrating mass shoots up into the air on a delicate stalk made up of the anterior cells which now have become vacuolated and encased in a hard cellulose sheath, and at the tip of this stalk there is a globular spore mass made up of cells encased in elliptical spore capsules. The range of individual cell sizes of each of these stages (with the exception of the mature stalk cells which could not be measured accurately because of their irregular shape) was determined and it was found that changes in the stage of development as well as early signs of differentiation were all reflected in characteristic changes of individual cell sizes.

## MATERIALS AND METHODS

The method of culture and the culture medium were the same as those used previously and the aggregating and migrating amoebae were prepared by centrifuging the vegetative amoebae free of bacteria and placing them on plain agar (Bonner, 1947). The cells at each stage were removed and placed in a drop of standard solution (NaCl, 0.60 gm.; KCl, 0.75 gm.; $CaCl_2$, 0.30 gm.; distilled $H_2O$, 1000 ml.) on a microscope slide. A No. 1 coverslip (22 × 22 mm.) was placed over the drop, but supported on two bits of coverslip so as to prevent the cells from being crushed. The diameters of the rounded, spherical cells were then measured with a 95 X, oil immersion objective and a Zeiss filar micrometer. In the case of the spores, which are elliptical in shape, both the long and the short axes were measured, and the spore diameter is expressed as the mean of these two values for each spore measured.

A number of tests were run to determine if the diameter of an individual cell remained constant when immersed in standard solution. It was found that during a period from 5 to 90 minutes after immersion the cell diameter did remain fairly constant, the small fluctuations being apparently caused by the activity of the contractile vacuole.

---

[1] This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.

The accuracy is considered to be most reasonable for the vegetative amoebae and the spores. However, during the aggregation and migration stages a fair per cent of the cells either did not round up after being subjected to the salt solution, or they remained part of a mass of cells and were impossible to measure. Therefore, during these two stages, there might conceivably be a sizable error due to having selected only those cells that were spherical. In defense of this it can only be said that there was no apparent or striking size difference and that it is believed that if an error has been introduced by this procedure it is likely to be relatively small.

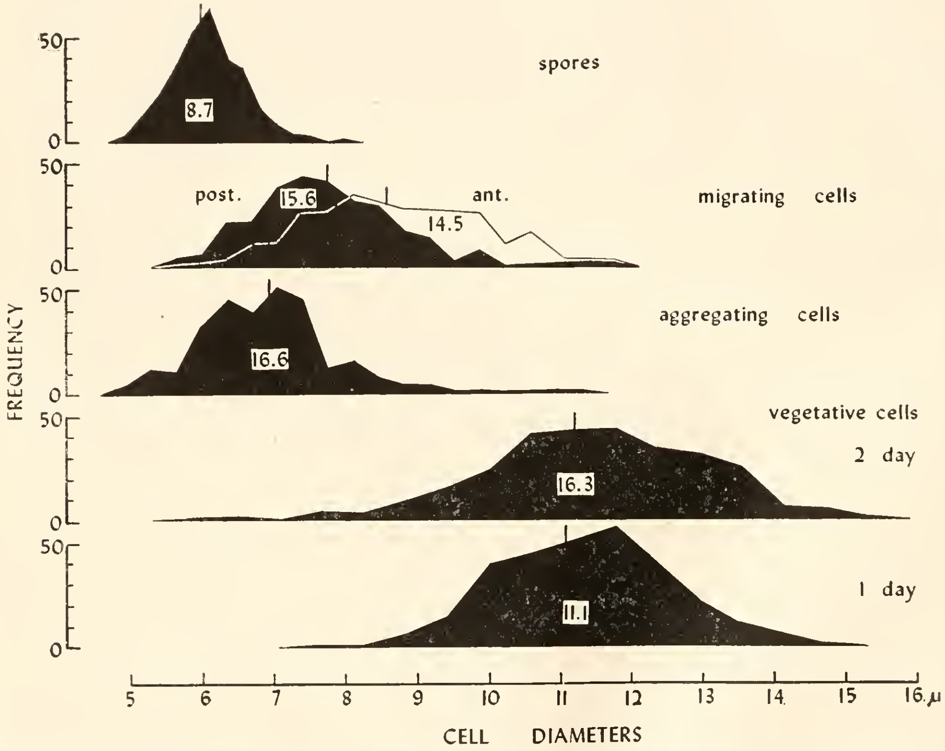


FIGURE 1. A graph in which the frequency is plotted against the cell diameter for various stages of development. Each curve consists of 300 observations. The vertical lines indicate the mean values. The numbers placed within the curves are the coefficients of variation in per cent.

## RESULTS

The diameters of 300 cells were measured for the following stages of development: (1) vegetative amoebae approximately one day old (between 22.5 and 23.5 hrs.), (2) vegetative amoebae approximately two days old (between 41.5 and 46 hrs.), (3) aggregating amoebae, (4) and (5) the anterior and posterior cells at the beginning of the migration stage (3 to 4 mm. of migration) and (6) the mature spores. For each stage, this count of 300 was made on three separate samples of 100, except in the case of the migrating cells which were measured in four samples (anterior) and five samples (posterior).

An analysis was made of the measurements at each of these stages to determine which of the three quantities, diameter, surface area or volume, would give a more normal distribution when plotted against frequency. It was found, by plotting the cumulative relative frequency against these three quantities on probability paper, that for all of the stages of development measured, the diameters gave the curve with the least skewness.[2] In fact the two vegetative amoëbae curves as well as the anterior migrating cells and the spores very closely approximated the normal distribution curves, while the aggregating cells and the posterior migrating cells showed a certain skewness in the higher end of their range.

If the frequencies are plotted against diameters for all the different stages and are shown on one graph, as has been done in Figure 1, it becomes obvious that the mean size (indicated by the vertical lines) goes through considerable fluctuations during the course of development. It remains constant throughout the vegetative period but makes a dramatic drop during the aggregation stage. During the migration period, the cells increase in size, but most interesting here is the fact that the anterior presumptive stalk cells are highly significantly larger than the posterior presumptive spore cells. A spot check was made on 50 anterior and 50 posterior cells of an old migrating cell mass (one that had migrated 25 mm.) and it was found to be the same as the young cell masses tested. The spores, on the other hand, are the smallest of all the stages.

In glancing at Figure 1, one can see that the range varies for each stage and an analysis was made to see to what extent this was related to size, for the larger the mean, the larger would be the expected range if the amount of variation about the mean were the same. To compare the different stages the usual procedure of dividing the standard deviation by the mean was employed to obtain the coefficient of variation. These coefficients are indicated on Figure 1 and it can be seen that during the first day of vegetative growth the variability is low but that it rises during the second day and remains high in the migration stage, only to drop to its lowest value in mature spores.

## Discussion

It is not surprising to find that the mean size does not change between one and two days of vegetative growth, and the cause of the sudden drop during aggregation is perhaps understandable for the vegetative amoebae are actively feeding and engulfing bacteria, while aggregating cells have been fasting for a considerable period of time. (In this case it has been some 17 to 18 hours since the amoebae were washed free of most of the bacteria by centrifugation.) There is no known explanation why the cells increase in size during the migration stage, but certainly it cannot involve feeding but must involve internal osmotic changes. The most important fact is that in the migrating cell mass the anterior presumptive stalk cells are significantly larger than the posterior presumptive spore cells, giving another example of an early detectable difference in differentiation. These migrating cell masses were stained with vital Nile blue sulfate and show characteristic dark anterior ends and light posterior ends (Bonner, 1952).

---

[2] The authors are greatly indebted to Dr. M. H. Belz, visiting Professor at the Department of Mathematics, Princeton University, for his help in the statistical work in this paper.

The variability during certain stages is remarkably high in *Dictyostelium* when one compares it to the variability of various unicellular organisms listed by Adolph (1931). To fully appreciate the consequences of this, one need only visualize the volumes; the largest two-day old vegetative amoebae, for instance, may be 18 to 19 times greater in volume than the smallest ones.

In comparing the coefficients of variation of the different stages there is an interesting trend, for the variability is low during the spore stage and rises somewhat after one day of vegetative existence, but only reaches a peak of variability after two days.

It is a curious fact that for these cell populations the diameters should give a normal distribution curve rather than the surface areas or volumes. Of course, since the factors which determine size, variability and skewness are virtually unknown, there is no reason to expect any particular type of distribution curve. An intriguing possibility might be, however, that the linear dimension gives a normal distribution because a ratio of the volume to the surface is in some way limiting to cell size and this ratio is, of course, linear. But unfortunately there is at the moment no way to weigh down such a wild speculation with a few facts.

## SUMMARY

1. The individual cell size was measured for 300 cells at various stages during the development of the slime mold *Dictyostelium discoideum;* during two periods of the vegetative stage when the amoebae are actively feeding, during the aggregation stage when the amoebae are streaming together to form cell masses, during the migration stage when the cell mass wanders over the substratum, and finally the mature encapsulated spores.

2. The mean size was large during the vegetative period, dropped severely in the fasting aggregating amoebae, increased slightly during the migration stage, only to fall to their minimum size as mature spores.

3. The variability in size was large and especially so during the periods before, during and after aggregation.

4. Of special interest relative to the problem of differentiation was the fact that during the migration stage, the anterior presumptive stalk cells were significantly larger than the posterior presumptive spore cells, being another example of an early detectable sign of differentiation.

## LITERATURE CITED

Adolph, E. F., 1931. The regulation of size as illustrated in unicellular organisms. C. C. Thomas, Baltimore.

Bonner, J. T., 1944. A descriptive study of the development of the slime mold *Dictyostelium discoideum. Amer. J. Bot.,* **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum. J. Exp. Zool.,* **106**: 1–26.

Bonner, J. T., 1952. The pattern of differentiation in amoeboid slime molds. *Amer. Nat.,* **86**: 79–89.

Raper, K. B., 1935. *Dictyostelium discoideum,* a new species of slime mold from decaying forest leaves. *J. Agric. Res.,* **50**: 135–147.

Raper, K. B., 1951. Isolation, cultivation and conservation of simple slime molds. *Quart. Rev. Biol.,* **26**: 169–190.

# RELATIONS BETWEEN PRE- AND POST-ANAEROBIC OXYGEN CONSUMPTION AND OXYGEN TENSION IN SOME FRESH WATER SNAILS

THEODOR VON BRAND AND BENJAMIN MEHLMAN [1]

*Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute,[2] Bethesda, Maryland*

Aquatic snails cannot be considered as particularly well adapted to anaerobic life since, in the absence of oxygen, they eventually lose their motility and die. They can, however, tolerate anaerobiosis for longer or shorter periods, these periods varying with the external temperature and also with the species (Jatzenko, 1928; Alsterberg, 1930; von Brand, Baernstein and Mehlman, 1950). Furthermore, it has been shown that some of the end products resulting from the anaerobic carbohydrate breakdown accumulate in the tissues (Mehlman and von Brand, 1951).

Animals giving this general type of response to lack of oxygen usually show in a subsequent aerobic recovery period a temporarily increased rate of oxygen consumption, a phenomenon variously known as repayment of an oxygen debt, respiratory rebound, or respiratory overshoot. Very little is known about this phase of snail physiology, and the few data available are contradictory. Borden (1931) found a very marked respiratory rebound in *Planorbis corneus,* while Füsser and Krüger (1951) did not find in the post-anaerobic respiration of this species and that of *Lymnaea stagnalis* a significant increase over the pre-anaerobic level.

Particular attention is paid in the present paper to the question of the relation between oxygen tension and pre- and post-anaerobic rates of oxygen consumption. It has been shown in many other invertebrates that the post-anaerobic oxygen consumption is frequently more dependent on the tension than the pre-anaerobic one. It has been claimed on the one hand that this indicates the participation of a different enzyme mechanism during the recovery period (Harnisch, 1935, 1936, 1951), while on the other hand von Buddenbrock (1939) considers the phenomenon as a necessary consequence of the increased respiratory level which would change the critical point at which diffusion ceases to furnish the organisms all the required oxygen. It has been pointed out previously (von Brand, 1947) that an extensive investigation on some non-parasitic invertebrate would be necessary to clarify the situation.

## MATERIALS AND METHODS

The following species of snails were used:

Planorbidae: *Australorbis glabratus,* laboratory-reared from Venezuelan stock, and *Helisoma duryi,* collected at Kenilworth, Maryland, but maintained in labora-

---

[1] The authors wish to express their appreciation for the contribution of snails to Mrs. M. O. Nolan, Mr. W. B. DeWitt and Miss B. Landry of this Laboratory and Dr. L. E. Noland of the University of Wisconsin, to Mr. N. Mantel for statistical advice, to Dr. J. B. Buck, Dr. L. Hellerman and Dr. June F. Zimmerman for constructive criticisms of the manuscript.

[2] Laboratory of Tropical Diseases.

tory aquaria for at least a week before being used. The Planorbidae employed ranged in weight from about 150 to 350 mg.

Physidae: Laboratory-reared *Aplexa nitens* from Texan stock, weighing from 200 to 500 mg.

Lymnaeidae: *Lymnaea stagnalis,* laboratory-reared, colony derived from laboratory-reared specimens obtained from Dr. L. E. Noland, University of Wisconsin. Juveniles weighing from about 100 to 600 mg. were used.

All respiration experiments were done by means of the conventional Warburg technique; vessels of about 16 ml. capacity were used, and 2 ml. of dechlorinated tap-water served as medium. Each specimen was used only for one experiment. The determinations were done at 30, 20 and 10° C. In general, a single snail was placed in a Warburg flask, but at 10° C. two snails frequently were used in order to obtain manometric readings of sufficient magnitude.

The usual experimental procedure was as follows: The rate of oxygen consumption was first established for 2.5 to 4 hours under atmospheric air (21 per cent oxygen). The gas phase was then changed, if required, by flushing the manometers with 100 per cent oxygen, 11 or 5 per cent oxygen in nitrogen (gases taken from steel cylinders; composition verified by analysis) and the respiratory rate again determined during 3 to 4 hours. Anaerobiosis was then established by flushing the manometers for 15 to 25 minutes with pure Linde nitrogen (99.99 per cent, further purified by passing over heated copper, pressure difference in manometer ca. 100 mm. Brodie fluid), and anaerobic conditions were maintained for 16 ± 0.5 hours. Shaking of the manometers was suspended after anaerobiosis was established since during this period no readings were taken. At the end of the 16 hours the vessels were removed from the manometers, the medium was withdrawn by means of a Pasteur pipette and replaced by fresh medium. In this way the excreted end-products were eliminated. They could otherwise have been reabsorbed or served as substrate for bacterial development during the recovery period. The manometers were then returned to the water bath and flushed with the same gas which had been used immediately preceding the anaerobic period. This flushing period served at the same time as temperature re-equilibration period. Readings were then taken usually for 8 hours, but in a few cases only for 7 or 7.5 hours.

Both pre- and post-anaerobic readings were taken at 15-minute intervals, but in experiments with *Australorbis glabratus* and *Helisoma duryi* at 10° C. the interval was 30 minutes. The data for each reading interval of each experiment were calculated separately. They were averaged and graphed for a given series. Owing to the many readings available for each experimental period, the respiratory pattern could be established with considerable accuracy. The experimental procedure permitted the direct comparison of the post-anaerobic oxygen consumption with the pre-anaerobic rate in both 21 per cent oxygen and in the same gas that was used immediately preceding the anaerobic period. In a few experiments, the pre-anaerobic relation between oxygen consumption in 21 per cent oxygen and the other tensions was established separately. Where in such cases post-anaerobic determinations were done, different snails were used and only one of the four gas mixtures was used both pre- and post-anaerobically.

TABLE I

*Influence of oxygen tension on the pre- and post-anaerobic oxygen consumption of well-fed snails*

| Species | Temp. °C. | No. of expts. | Pre-anaerobic rates | | | Post-anaerobic maximal rate | |
|---|---|---|---|---|---|---|---|
| | | | Rate in 21 per cent $O_2$ mm.$^3O_2$/gm./hr. | $O_2$ per cent in exp. gas | Rate in exp. gas in per cent of rate in 21 per cent $O_2$ | In per cent of pre-anaerobic rate in 21 per cent $O_2$ | In per cent of pre-anaerobic rate in exp. gas |
| *Australorbis glabratus* | 30 | 17 | 119.0±6.4 | 100 | 109±5.0 | 170±6.3 | 157±7.0 |
| *Australorbis glabratus* | 30 | 16 | 120.9±5.2 | 21 | 100 | 162±10.3 | 162±10.3 |
| *Australorbis glabratus* | 30 | 14 | 148.7±8.8 | 11 | 104±5.7 | 147±7.8 | 141±7.5 |
| *Australorbis glabratus* | 30 | 31 | 144.8±6.4 | 5 | 66±4.6 | 89±3.7 | 135±5.6 |
| *Australorbis glabratus* | 20 | 12 | 50.2±2.9 | 100 | 114±6.4 | 151±9.9 | 133±5.0 |
| *Australorbis glabratus* | 20 | 18 | 69.2±2.8 | 21 | 100 | 122±4.7 | 122±4.7 |
| *Australorbis glabratus* | 20 | 12 | 56.0±6.0 | 11 | 86±9.6 | 142±13.3 | 173±13.1 |
| *Australorbis glabratus* | 20 | 11 | 62.7±5.0 | 5 | 69±6.8 | 80±7.7 | 122±13.9 |
| *Australorbis glabratus* | 10 | 20 | 18.8±0.6 | 100 | 98±3.9 | 148±6.2 | 154±9.8 |
| *Australorbis glabratus* | 10 | 18 | 11.1±0.4 | 21 | 100 | 130±7.4 | 130±7.4 |
| *Australorbis glabratus* | 10 | 22 | 18.6±1.1 | 11 | 77±2.4 | 134±6.7 | 178±11.2 |
| *Australorbis glabratus* | 10 | 20 | 17.4±0.6 | 5 | 66±3.9 | 107±7.2 | 170±18.3 |
| *Australorbis glabratus** | 20 | 14 | 52.0±3.7** | 100 | 114** | 189** | 170±6.9 |
| *Australorbis glabratus** | 20 | 17 | 58.6±4.8 | 21 | 100 | 145±8.0 | 145±8.0 |
| *Australorbis glabratus** | 20 | 13 | 72.7±3.8** | 11 | 86** | 120** | 143±10.7 |
| *Australorbis glabratus** | 20 | 21 | 59.3±2.4** | 5 | 69** | 94** | 142±7.3 |
| *Helisoma duryi* | 30 | 15 | 125.9±13.2 | 100 | 111±6.2 | 211±13.6 | 192±12.4 |
| *Helisoma duryi* | 30 | 16 | 137.3±3.4 | 21 | 100 | 156±6.0 | 156±6.0 |
| *Helisoma duryi* | 30 | 14 | 185.9±10.5 | 11 | 81±4.1 | 138±7.2 | 170±8.9 |
| *Helisoma duryi* | 30 | 14 | 181.8±4.7 | 5 | 50±4.0 | 72±3.3 | 152±6.6 |
| *Helisoma duryi* | 20 | 14 | 56.2±3.6 | 100 | 93±4.3 | 141±9.5 | 154±9.5 |
| *Helisoma duryi* | 20 | 13 | 49.2±5.2 | 21 | 100 | 166±10.0 | 166±10.0 |
| *Helisoma duryi* | 20 | 13 | 49.3±3.4 | 11 | 83±3.0 | 132±12.4 | 157±8.3 |
| *Helisoma duryi* | 20 | 12 | 45.6±4.6 | 5 | 64±3.0 | 112±8.3 | 179±13.7 |
| *Helisoma duryi* | 10 | 16 | 15.5±1.0 | 100 | 87±6.9 | 197±16.0 | 232±16.6 |
| *Helisoma duryi* | 10 | 16 | 13.7±0.7 | 21 | 100 | 148±9.5 | 148±9.5 |
| *Helisoma duryi* | 10 | 16 | 13.2±1.0 | 11 | 93±5.9 | 138±13.0 | 155±13.4 |
| *Helisoma duryi* | 10 | 16 | 15.0±0.4 | 5 | 79±3.6 | 168±18.3 | 217±18.3 |
| *Aplexa nitens* | 20 | 12 | 50.3±4.2 | 100 | 126±6.6 | 175±13.3 | 139±9.7 |
| *Aplexa nitens* | 20 | 12 | 59.9±7.0 | 21 | 100 | 154±11.6 | 154±11.6 |
| *Aplexa nitens* | 20 | 16 | 62.6±1.7 | 11 | 85±7.5 | 133±4.5 | 156±5.7 |
| *Aplexa nitens* | 20 | 9 | 42.1±4.5 | 5 | 74±9.9 | 144±23.1 | 186±31.6 |
| *Lymnaea stagnalis* | 20 | 14 | 150±7.6 | 100 | 93±4.7 | 128±6.0 | 139±7.1 |
| *Lymnaea stagnalis* | 20 | 15 | 84.9±9.1 | 21 | 100 | 115±4.6 | 115±4.6 |
| *Lymnaea stagnalis* | 20 | 16 | 149.6±12.3 | 11 | 73±3.2 | 69±6.6 | 95±8.1 |
| *Lymnaea stagnalis* | 20 | 14 | 109.5±9.4 | 5 | 52±2.8 | 54±5.8 | 105±10.0 |
| *Lymnaea stagnalis* | 10 | 17 | 41.8±1.3 | 100 | 96±3.2 | 117±1.1 | 124±6.2 |
| *Lymnaea stagnalis* | 10 | 17 | 35.4±1.3 | 21 | 100 | 122±9.2 | 122±9.2 |
| *Lymnaea stagnalis* | 10 | 16 | 35.3±2.0 | 11 | 91±4.8 | 106±5.3 | 116±3.6 |
| *Lymnaea stagnalis* | 10 | 17 | 34.2±2.3 | 5 | 60±3.5 | 74±5.0 | 126±6.8 |

The oxygen determinations were done at the same temperature used for the anaerobic exposure in all series, except those marked with an asterisk. In these the oxygen determinations were carried out at 20° C., but the snails were exposed to anaerobiosis at 30° C. In this series only the experimental gas was used pre-anaerobically; the values marked by two asterisks are calculated on the assumption that the pre-anaerobic oxygen consumption showed the same dependency on the tension as shown by the corresponding series done with the same species at the same temperature and shown elsewhere in this table. All averages are arithmetic averages and hence show a slight upward bias. The figures after the ± signs are the standard error of the mean.

## RESULTS

### 1. *Oxygen tension and pre-anaerobic oxygen consumption*

A study of the pre-anaerobic rates of oxygen consumption of *Australorbis glabratus* (Tables I and II) shows that in this species the percentage decline in oxygen consumption at tensions below 21 per cent oxygen was on the whole remarkably similar regardless of whether the metabolic rate was changed by using different temperatures or snails of different nutritional state. One curious observation was that the oxygen consumption remained virtually unaffected by lowering the oxygen concentration from 21 to 11 per cent at 30° C., while the rate was definitely lowered under analogous conditions at 20° and 10° C. It is not believed that this somewhat paradoxical finding is to be attributed to experimental errors because in previous experiments (von Brand, Nolan and Mann, 1948) a similar, or even somewhat more pronounced independence of the oxygen consumption on the tension had been found at 30° C.

TABLE II

*Influence of starvation on the dependency of the pre-anaerobic oxygen consumption on the oxygen tension. The rates are expressed in mm.³ oxygen per gm. fresh weight per hour*

| Species | Temp °C. | No. exp. | Starvation days | Oxygen consumption in per cent oxygen | | 5 per cent rate in per cent of 21 per cent rate |
|---|---|---|---|---|---|---|
| | | | | 21 | 5 | |
| *Australorbis glabratus* | 30 | 31 | 0 | 144.8±6.4 | 95.7±4.2 | 66±4.6 |
| *Australorbis glabratus* | 30 | 17 | 14 | 64.1±1.4 | 46.7±2.5 | 73±3.7 |
| *Australorbis glabratus* | 20 | 11 | 0 | 62.7±5.0 | 41.3±3.6 | 69±6.8 |
| *Australorbis glabratus* | 20 | 23 | 14 | 34.2±1.3 | 22.7±1.8 | 66±4.2 |
| *Helisoma duryi* | 30 | 14 | 0 | 181.8±4.5 | 90.9±4.7 | 50±4.0 |
| *Helisoma duryi* | 30 | 12 | 14 | 55.6±6.3 | 42.4±4.0 | 78±2.5 |
| *Helisoma duryi* | 20 | 12 | 0 | 45.6±4.6 | 27.9±2.3 | 64±3.0 |
| *Helisoma duryi* | 20 | 10 | 14 | 29.4±1.9 | 20.4±1.6 | 73±7.1 |

In *Helisoma duryi* and *Lymnaea stagnalis,* it is evident that lowering the metabolic rate did lead to a reduction in the dependency of the respiratory rate on the tension, although the effect was not very pronounced.

In view of these variations it is difficult, if not impossible, to grade the snail species used as to the magnitude of influence of oxygen tension. Apparently only minor differences exist in this respect between the four species investigated and, within the range of tensions tested, the species with hemoglobin in their blood (*Australorbis* and *Helisoma*) are, at a given temperature, capable of securing only a slightly higher percentage of their maximal oxygen needs than the snails having hemocyanin (*Lymnaea* and *Aplexa*). This is in approximate agreement with the findings of Füsser and Krüger (1951) on *Planorbis* and *Lymnaea.* We are also in agreement with these workers as to the finding that the actual respiratory rate, at equal temperature, is higher in *Lymnaea* than the other species. Evidently, the temperature relationships of various snail species must vary, since in previous experiments no such difference was found at 30° C. (von Brand, Nolan and Mann, 1948).

### 2. *Post-anaerobic respiration*

All four snail species studied showed a marked respiratory rebound after having been exposed to 16 hours of anaerobiosis (Table I). In all cases the period of excess oxygen consumption lasted a long time and three different types of recovery curves could be distinguished. In type 1, characteristic for *Helisoma,* the post-anaerobic rate was higher than the pre-anaerobic level immediately after restoration of aerobic conditions (excluding, of course, the time necessary for changing the medium and the other necessary manipulations, for which no data are available). The rate remained at a high level for several hours and then began to approach the normal without, however, reaching it completely within the time of observation. In type 2, occurring frequently in *Australorbis* and *Lymnaea,* the oxygen consumption was increased from the beginning and remained at this same level for the entire period of 7 to 8 hours. In type 3, observed in some series of all snails and the only one seen in *Aplexa,* the post-anaerobic rate was initially relatively low. After a period, varying from about one to three hours, a transition to maximal respiratory activity (higher than pre-anaerobic) occurred and this rate was usually sustained for the balance of the observation period. In a few cases, especially with *Australorbis,* a distinct lowering of the rate occurred during the 7th or 8th hour. During the initial period, the oxygen consumption was either somewhat lower or higher than the pre-anaerobic rate, but in most instances it was surprisingly close to the pre-anaerobic level, giving, perhaps erroneously, the impression that the normal (pre-anaerobic) metabolism was resumed immediately after restoration of aerobiosis, but that a certain induction period was necessary in these cases to initiate the actual respiratory rebound.

It is evident that owing to the long periods of "repayment" no definite data can be given concerning the total amount of oxygen repaid. However, in those cases where, after an initial high level, the post-anaerobic rate later approached the pre-anaerobic rate, it can be stated with some certainty, on the basis of a few observations made after 24 hours, that the repayment was far from complete. This is in contrast to the findings of Borden (1931) on *Planorbis corneus.* Borden used only very short periods of anaerobiosis; we used long periods. It is very probable that the proportion between anaerobic end-products accumulating in the tissues and being excreted varies under such different conditions and that this factor may be responsible for the differences in results. In our opinion the significance of the term "complete repayment of an oxygen debt" is sometimes over-emphasized. Insofar as invertebrates are concerned it does not imply anything of fundamental importance. The total amount of oxygen repaid will depend primarily upon the level of anaerobic metabolism, the nature of the anaerobic end-products, and the question to what extent they are excreted or stored in the tissues. None of these factors has necessarily a close quantitative connection with the pre-anaerobic oxidative metabolism, that is, with the amount of oxygen missed during anaerobiosis, but they will decide whether the post-anaerobic overshoot leads to an incomplete or complete repayment of the oxygen debt, or even to a more or less marked overpayment (review of the pertinent literature in von Brand, 1946).

Special attention was given to the relationship existing between pre-anaerobic rate of oxygen consumption and maximal post-anaerobic rate sustained over at least two hours and usually over a much longer period. A study of Table I shows that in most series the post-anaerobic respiration was more dependent on the oxygen tension than the pre-anaerobic one if the pre-anaerobic consumption at 21 per cent oxygen is taken as reference point for both. It is also evident that changes in the metabolic level induced by temperature changes have no decisive influence on the relation between pre- and post-anaerobic rates of oxygen consumption. The variations between the various series conducted with one and the same

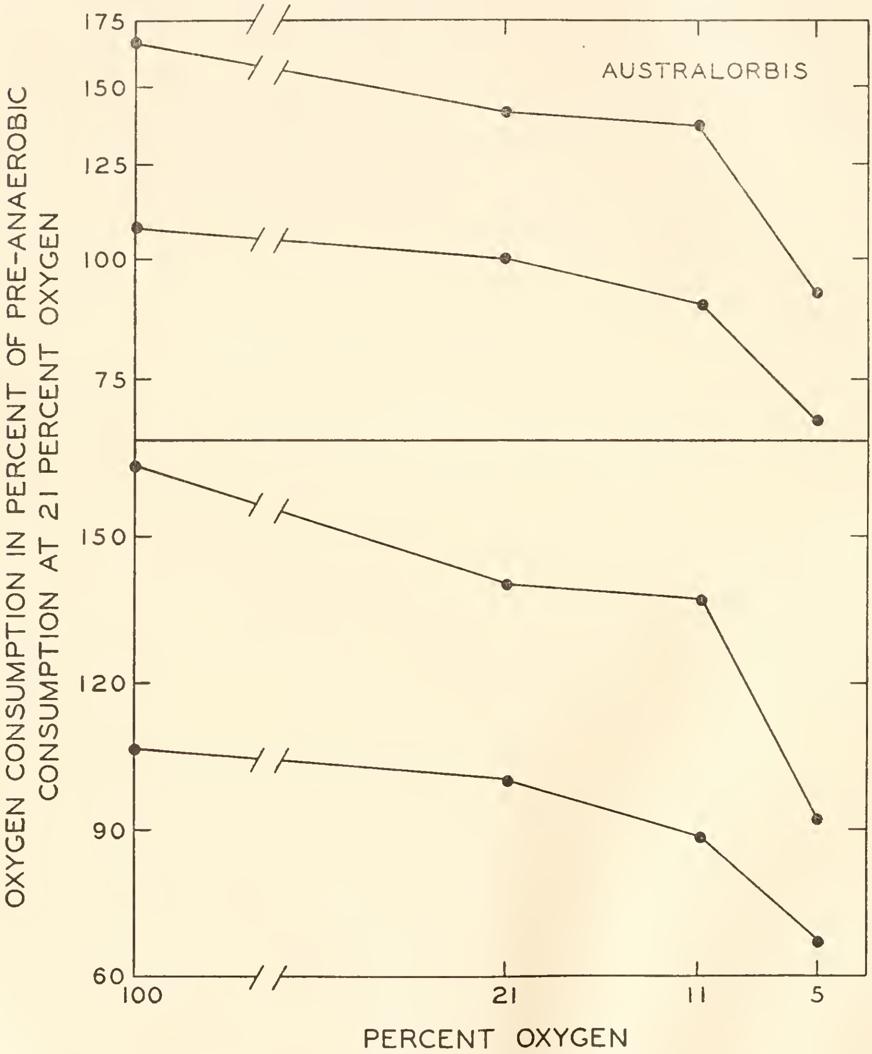


FIGURE 1. Dependency of the pre- and post-anaerobic respiration of *Australorbis glabratus* on the oxygen tension. Average values of all series plotted on an arithmetic scale (lower part of figure) and logarithmic scale (upper part of figure).

snail species are fairly large, but this will surprise no one who has had experience with metabolic studies on this group of organisms.

Undoubtedly, some differences exist in the pre- to post-anaerobic ratios for the various conditions considered which appear significant from a purely statistical standpoint. By and large, however, the ratios are in the same region at all temperatures studied. Moreover, no constant pattern of temperature differences could be found when different oxygen tensions were used. Presumably, the significant differences reflect differences in the various batches of snails rather than differences due to temperature or oxygen tension. Separate batches of snails had to be used for each series and the entire experiments spread out over about a year.

As mentioned in a previous section the percentage decline in pre-anaerobic respiration at lowered oxygen tensions is unaffected by temperature changes only in *Australorbis.* It appears, therefore, permissible only in this species to average all the available series in order to minimize the biological variations by using the greatest possible number of observations. The results are shown in Figure 1. When plotted on an arithmetic scale, as has been done heretofore by all investigators, the greater dependency of the post-anaerobic oxygen consumption on the oxygen tension is quite evident. If considered from this standpoint alone, similar findings of Harnisch (1935, 1936) on insects and worms are apparently confirmed. If, however, the data are plotted on a logarithmic scale, a different picture emerges. The lines for the pre- and post-anaerobic respiration now rather closely parallel each other, indicating that the percentage increase of the post-anaerobic respiration over the pre-anaerobic rate is approximately identical at all tensions studied. The deviations of the two lines upon plotting on an arithmetic scale are quite apparently attributable to rate differences sustained by the pre-anaerobic respiration at the various oxygen tensions. Therefore, they actually do not constitute a proof for the contention that the post-anaerobic respiration is more dependent on the tension; rather, they prove the contrary.

The situation is essentially the same in the other snail species investigated. Again, considerable differences within series exist (Table I). The trend of the figure, however, is unmistakable. In all species, the post-anaerobic oxygen consumption is raised over the pre-anaerobic level to about the same degree regardless of the oxygen tension. A survey of Table I shows that *Helisoma* raised its post-anaerobic level highest, that of *Lymnaea* remained always relatively low, and the two other species were intermediate.

## DISCUSSION

The present investigation has shown that the oxygen consumption of four species of pulmonate snails is somewhat dependent on the tension below 21 per cent oxygen and that this dependency shows little connection with the actual rate of oxygen consumption. In *Australorbis glabratus* held in 5 per cent oxygen, the percentage decline (around 34 per cent) was the same whether (1) the actual respiratory level in 21 per cent oxygen was reduced from 145 $mm.^3/gm./hr.$ to 17 $mm.^3$ by changing the temperature from 30° C. to 10° C., or (2) from 145 $mm.^3$ to 64 $mm.^3$ by starvation. In *Lymnaea stagnalis* and *Helisoma duryi*, the dependency was not quite as unalterable. In the latter species, for instance, the percentage decline in 5 per cent oxygen was 50 per cent when the metabolic level was 182 mm.$^3$/gm./hr. in 21 per cent oxygen, 36 per cent at a level of 46 mm.$^3$ and 21 per cent at a level of 15 mm.$^3$. It is evident that even in this latter case the oxygen consumption does not entirely conform to what would be expected if diffusion were the only limiting factor. According to the diffusion hypothesis, which at present seems to be accepted almost universally, lowering the oxygen tension below a critical point leads to a lowering of the over-all consumption because at lower tensions all the oxygen entering the body by diffusion is consumed by the superficial tissue layers and an internal layer becomes anoxic. Since temperature has but little influence on the rate of diffusion and starvation probably has none,[3] it would appear that the oxygen consumption of the above snails held in 5 per cent oxygen at 10° C. should have been about 100 per cent of that shown in air if the diffusion hypothesis held strictly. This seems to follow from the observation that at 30° C. in 5 per cent oxygen diffusion was able to bring much more oxygen into the body than the snails consumed at 10° C. in 21 per cent oxygen.

The above argument is not quite straightforward, however, because the snails have a circulatory system. It is conceivable that the oxygen transport within the body and/or the influence of temperature on the loading and unloading tension of the respiratory pigments may be the limiting factors. In order to shed some light on this point, several observations on the rate of heart-beat were done. In 15 *Australorbis* it was reduced at 10° C. to 13 per cent of the 30° rate, while the rate of oxygen consumption was reduced to 11 per cent. Similar experiments with 6 *Lymnaea* showed at 10° C. reductions in both rates corresponding to 44 and 43 per cent, respectively, of the 20° C. rate. This close parallelism between the rates of heart-beat and oxygen consumption tends therefore to support the above view. However, no such close parallelism was found in respect to the influence of starvation. A starvation period of two weeks reduced the rates of heart-beat and oxygen consumption of 15 *Australorbis* to 75 and 44 per cent of the pre-starvation rates. It seems therefore that another hitherto neglected factor may be of greater importance than believed in recent years: namely, the oxygen tension as such. It is of interest in this connection to note that Füsser and Krüger (1951) concluded from their study of normal and carbon monoxide-poisoned *Planorbis corneus* and *Lymnaea stagnalis* that the diffusion hypothesis alone does not suffice to explain the oxygen consumption-oxygen tension relationships. They assume a direct influence of the oxygen concentration but do not elaborate the possibility in greater detail. We shall return to this point after having discussed the post-anaerobic respiration of our objects.

All four species of snails used in the present study showed a respiratory rebound and in all species it was characterized by its long duration. In this respect the snails resembled the clam *Sphaerium corneum* (Jatzenko, 1928). In some other invertebrates, such as insects and worms, the repayment period lasts a shorter time, but the percentage increase of the post-anaerobic respiration may be appreciably higher (Harnisch, 1936, 1948; von Brand, 1947).

The post-anaerobic oxygen consumption of the snails appeared more dependent on the tension than the pre-anaerobic consumption when expressed in absolute terms or in per cent of the pre-anaerobic consumption in 21 per cent oxygen. In this respect the present findings parallel those of Harnisch (1936), but they do not necessarily support his conclusions. In his opinion the above phenomenon indicates that normal and excess respiration are mediated through different enzyme systems. However, our data show that in aquatic snails the post-anaerobic oxygen consumption is raised over the pre-anaerobic rate by approximately the same percentage, regardless of the tension employed. This apparently hitherto unrecognized relationship leads inevitably to the picture of greater dependency of the rebound respiration on the tension than that shown by the normal respiration, if expressed in absolute terms or in per cent of the pre-anaerobic value shown in 21 per cent oxygen. It is simply a consequence of the fact that increasing a graded series of figures by the same percentage makes the difference between elevated and base figure the greater, the greater the magnitude of the base figure is. Evidently, then, the oxygen tension/oxygen consumption relationship does not give any information as to whether different enzyme systems are responsible for the pre- and post-anaerobic oxygen consumption.

---

[3] It is realized that this statement may need revision if further evidence becomes available. If starvation reduces the enzyme content of the cells materially, or if one accepts the free-radical mechanism for enzymatic reactions, starvation may change diffusion.

Our data also obviously contradict von Buddenbrock's (1939) view that the greater dependency of the overshoot respiration is attributable to a shift in the critical point towards a higher oxygen tension owing to the increased rate shown in this period. However, our observations do raise some new questions which at present can be answered only tentatively.

The obvious first question is why the post-anaerobic respiration should be raised by the same percentage over a wide range of tensions. Apparently, the degree of anaerobic stress plays only a minor role. The percentage increase was, within admittedly rather wide limits of variations, similar regardless of the temperature at which the specimens of one species had been exposed for an equal period to anaerobiosis. There is general agreement that the overshoot phenomenon is due to the accumulation of end-products of anaerobic metabolism. The increase in substrates would lead to an increased probability of enzyme and substrate molecules colliding (Zimmerman, 1949). The long period of repayment seems to indicate that a regulatory mechanism exists in snails which releases the "abnormal" substrates only slowly to the oxidative processes. This process might be geared to the rate of oxidations involving the normal substrates. Such an assumption would explain the phenomena observed.

Another very interesting question is the explanation of the physiological mechanism by which snails and other organisms are able to raise their post-anaerobic respiratory rate at those tensions where the normal respiration declines. This quite common phenomenon seems, surprisingly enough, never to have been discussed. It is a curious phenomenon because it indicates a regulatory mechanism that comes into play only post-anaerobically but not when the snail is exposed pre-anaerobically to oxygen concentrations allowing only 50 to 60 per cent of the normal maximal rate of respiration. If diffusion of oxygen into the body were the only limiting factor pre-anaerobically, one would obviously have to look for factors that would facilitate a greater influx of oxygen post-anaerobically. Such factors may be an increased surface by maximal extension of the foot, a facilitated oxygen release by the blood due to a Bohr effect, a steeper oxygen gradient in the tissue layers adjacent to the diffusion surfaces owing to the accumulation of substrates, or due to an increased enzyme concentration. Such factors might well be important and possibly suffice to explain the phenomenon. Unfortunately, a highly organized animal, such as a snail, represents so complex a system that a complete analysis of these factors, experimentally or mathematically, would appear to be extremely difficult, if not impossible. It seems therefore appropriate to point out that at least one other alternative exists.

It is, for example, possible to discuss the present findings from a standpoint already touched upon above, namely, to raise the question whether the importance of the oxygen tension as such may not be greater than is usually suspected. Assuming that at least one enzymatic key-process of the cellular oxidation mechanism operates more efficiently at higher than at lower oxygen tensions, the following picture would appear. The relative inefficiency of a changed metabolic level in influencing the critical point of the pre-anaerobic oxygen consumption/oxygen tension relationship would be understandable without any auxiliary hypothesis since the influence of the tension would remain the same regardless of the temperature employed or the nutritional state of the snails. A necessary implication of this view would be that even at low tensions (low within limits, of course) the decrease of the pre-anaerobic oxygen consumption would not be due to the establishment of an anoxic zone in the deeper tissue layers. Even these tissues, like all the others, would get enough oxygen to allow a certain sub-optimal oxidative activity, the actual level of which would be determined on the one hand by the intracellular oxygen tension as influenced by the external tension, and on the other hand, by the availability of substrates. It may be pointed out in this connection that the fluoroacetate inhibition of citrate utilization in pigeon breast muscle is profoundly influenced by the oxygen tension (Massey and Rogers, 1951). Furthermore, Lehmann (1935) has found that succinic dehydrogenase was most active at oxygen tensions of 44 to 56 mm. Hg, while the activity significantly decreased at lower tensions. Finally, Schade and Levy (1949) have found in potato tissues, besides cytochrome oxidase, a terminal oxidase which was markedly sensitive to lowered oxygen tensions.

Insofar as post-anaerobic conditions are concerned, the increase in substrates and their gradual release to the oxidative processes would allow an increased oxygen consumption of all tissues and hence lead to an increase in the over-all oxygen consumption at all tensions where the above requirement is fulfilled, namely, that diffusion limitations would not be so severe as to lead to the establishment of an anoxic zone. The mechanism of the post-anaerobic increase in rate would hence be reduced to the accumulation of anaerobic metabolic end-products. The occurrence of the same percentage increase in post-anaerobic respiration over a wide range of tensions could also be understood better because it can be visualized that under otherwise identical conditions, the increased substrates and their gradual release may have, percentage wise, the same stimulatory effect on optimal and sub-optimal oxidative activity.

It should be understood that the above explanation is proposed only as a possible working hypothesis. It is realized that the available evidence does not constitute a definite proof. Furthermore, the above hypothesis is not mutually exclusive with the diffusion hypothesis. In any animal diffusion must become the limiting factor if the tension is lowered sufficiently. The critical tension will vary from species to species, probably being highest in bulky animals lacking a circulatory system, like the actinians. Conversely, it is indisputable that somewhere along the line of oxygen tensions a critical point must be reached for any oxidation process below which it cannot function with full effectiveness. Insofar as the snail species studied are concerned, it is probable that both components enter the picture. Least affected by diffusion difficulties and hence most subject to the direct influence of tension seems to be *Australorbis glabratus,* while in *Helisoma duryi* and *Lymnaea stagnalis* diffusion difficulties seem to play a greater role as evidenced by the influence of the metabolic level on the critical point.

## SUMMARY

1. The respiration of four species of fresh water snails was somewhat dependent on the oxygen tension below 21 per cent oxygen with only minor differences among the various species.

2. The degree of dependency was influenced little, if any, by alteration of the metabolic rate of *Australorbis glabratus,* but some changes were obtained in the cases of *Helisoma duryi* and *Lymnaea stagnalis.*

3. All species showed a long lasting respiratory rebound after 16 hours anaerobiosis. The post-anaerobic respiration was more dependent on the oxygen tension than the pre-anaerobic respiration if referred to the normal rate shown at 21 per cent oxygen. But if the post-anaerobic rates were compared with the rates sustained pre-anaerobically at an identical oxygen tension, an approximately equal percentage increase was observed over a wide range of tensions.

4. The implications of these observations and auxiliary observations dealing with the rate of the heart-beat under various conditions are discussed insofar as they shed light on the mechanism of the pre- and post-anaerobic respiration. It is concluded that diffusion alone cannot be the sole limiting factor that reduces the over-all oxygen consumption when the tension is lowered below a critical point, and the idea is discussed that the oxygen tension as such may be more important for certain cellular processes than usually assumed.

## LITERATURE CITED

ALSTERBERG, G., 1930. Wichtige Züge in der Biologie der Süsswassergastropoden. Gleerupka, Lund.

BORDEN, M. A., 1931. A study of the respiration and of the function of haemoglobin in *Planorbis corneus* and *Arenicola marina. J. Marine Biol. Assoc. United Kingdom N. S.,* **17**: 709–738.

VON BRAND, T., 1946. Anaerobiosis in invertebrates. Biodynamica Monographs No. 4, Biodynamica, Normandy, Missouri.

VON BRAND, T., 1947. Physiological observations upon a larval *Eustrongylides.* XI. Influence of oxygen tension on the aerobic and post-anaerobic oxygen consumption. *Biol. Bull.,* **92**: 162–166.

VON BRAND, T., H. D. BAERNSTEIN AND B. MEHLMAN, 1950. Studies on the anaerobic metabolism and the aerobic carbohydrate consumption of some fresh water snails. *Biol. Bull.,* **98**: 266–276.

VON BRAND, T., M. O. NOLAN AND E. R. MANN, 1948. Observations on the respiration of *Australorbis glabratus* and some other aquatic snails. *Biol. Bull.,* **95**: 199–213.

VON BUDDENBROCK, W., 1939. Grundriss der vergleichenden Physiologie. 2nd ed. Vol. 2. Gebruder Bornträger, Berlin.

FÜSSER, H., AND F. KRÜGER, 1951. Vergleichende Versuche zur Atmungsphysiologie von *Planorbis corneus* und *Limnaea stagnalis* (Gastropoda pulmonata). *Zeitschr. vergl. Physiol.*, **33**: 14–52.

HARNISCH, O., 1935. Versuch einer Analyse des Sauerstoffverbrauchs von *Tubifex tubifex* Müll. *Zeitschr. vergl. Physiol.*, **22**: 450–465.

HARNISCH, O., 1936. Primäre und sekundäre Oxybiose der Larve von *Chironomus thummi* (nebst ergänzenden Messungen an *Tubifex tubifex*). *Zeitschr. vergl. Physiol.*, **23**: 391–419.

HARNISCH, O., 1948. Untersuchungen zum Wesen der Erholungsatmung. *Zeitschr. Naturforschung,* **3 b**: 453–457.

HARNISCH, O., 1951. Hydrophysiologie der Tiere. Vol. 19 of: Thieneman, A., Die Binnengewässer. Schweizerbarth, Stuttgart.

JATZENKO, A. T., 1928. Die Bedeutung der Mantelhöhlenflüssigkeit in der Biologie der Süsswasserlamellibranchier. *Biol. Zentralbl.*, **48**: 1–25.

LEHMANN, J., 1935. Über den Sauerstoffverbrauch bei der vitalen Bersteinsäureoxydation in Abhängigkeit von pH und Sauerstoffdruck. *Skand. Arch. Physiol.*, **72**: 78–91.

MASSEY, V., AND W. P. ROGERS, 1951. Conditions affecting the action of fluoroacetate on the metabolism of nematode parasites and vertebrate animals. *Australian J. Sci. Research, Ser. B.*, **4**: 561–574.

MEHLMAN, B., AND T. VON BRAND, 1951. Further studies on the anaerobic metabolism of some fresh water snails. *Biol. Bull.*, **100**: 199–205.

SCHADE, A. L., AND H. LEVY, 1949. Studies on the respiration of the white potato. III. Changes in the terminal oxidase pattern of potato tissue associated with time of suspension in water. *Arch. Biochem.*, **20**: 211–219.

ZIMMERMAN, J. F., 1949. Absolute reaction rate theory and the respiratory rebound. *Biochem. Biophys. Acta,* **3**: 198–204.

# MITOTIC EFFECTS OF PROLONGED IRRADIATION WITH LOW-INTENSITY GAMMA RAYS ON THE CHORTOPHAGA NEUROBLAST

J. GORDON CARLSON, NYRA G. HARRINGTON AND
MARY ESTHER GAULDEN

*Biology Division, Oak Ridge National Laboratory,[1] Oak Ridge, Tennessee, and Department of Zoology and Entomology, University of Tennessee,[2] Knoxville, Tennessee*

When cells are subjected to given doses of ionizing radiation delivered at very low rates, the detectable effects on mitotic activity, as measured at the end of the treatment period, resemble those obtained from smaller doses administered at higher intensities (Carlson, Snyder and Hollaender, 1949; Carlson, 1950). This is due, apparently, to the capacity of living cells to recover from the more immediate physical and chemical changes induced by irradiation even while treatment is in progress. At the end of a short treatment period the reduced mitotic activity will represent virtually all the potential mitotic effect produced, because little recovery from these initial effects will have occurred. At the end of a long period of irradiation, on the other hand, the decreased mitotic activity will be the result of only the physico-chemical changes produced in the later part of the treatment period, those induced earlier having undergone recovery before the period of observation.

The main purpose of the present study was to investigate the mitotic effect of prolonged low-intensity treatment and to determine whether a balance between effect and recovery would be established and maintained at about the same level over much of the extended periods during which treatment was in progress. If such a balance were established, the detectable mitotic effects would be approximately the same at the ends of a wide range of treatment periods for a given dosage rate.

## MATERIALS AND METHODS

Eggs of *Chortophaga viridifasciata* (DeGeer) were used in all experiments. Because the times of treatment ranged from two to six days, it was necessary to start with embryos sufficiently young so that at the end of treatment they would be at the optimum age (14 days at 26° C.) for studying the mitotic activity of the neuroblasts.

The apparatus used in this study to irradiate the grasshopper eggs with $\gamma$ rays is shown in Figure 1. The source (B) consisted of activated cobalt-aluminum alloy in the form of a hollow cylinder 8 inches long and 2 inches in diameter with walls 0.03 inch thick. This was enclosed in a cylindrical lead pig (A), the walls and lid of which each measured 2 inches in thickness. A cylinder of polystyrene

---

[1] Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

[2] Contribution No. 71 from the Department of Zoology and Entomology of The University of Tennessee.

(C), bored from one end to a depth of 5½ inches with a ¾-inch bit, fitted snugly inside the source. Four polystyrene vessels (E), which were threaded so that they could be screwed to one another and to a polystyrene plug (D) held the eggs during treatment. The interior dimensions of each vessel, when screwed together, were approximately ½ inch in diameter and ¼ inch in depth. Treatment was begun by inserting the joined vessels and plug, vessels first, into the bore of the polystyrene cylinder (C). When in this position, the material to be treated was situated no more than one inch above or below the middle of the source, which extended at least three inches beyond the vessels at either end. Treatment was terminated by withdrawing the plug and attached vessels.

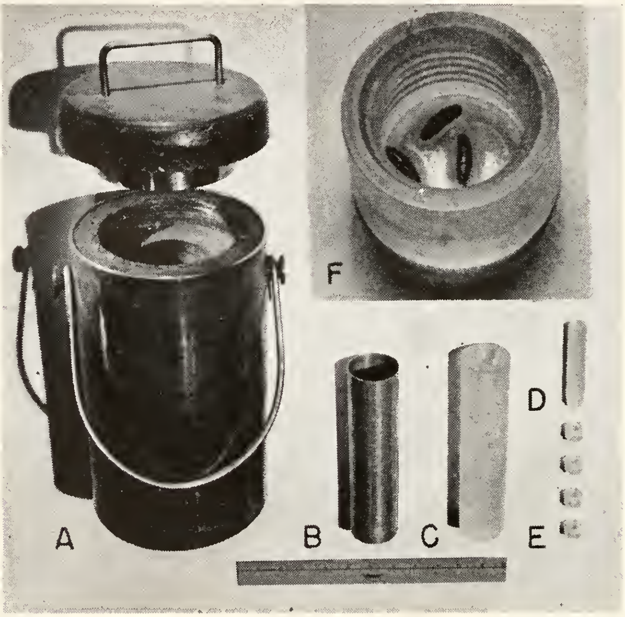


FIGURE 1. Gamma source and accessories. A, lead pig; B, cobalt-aluminum alloy source; C, polystyrene cylinder; D, polystyrene plug; E, polystyrene vessels; F, polystyrene vessel (enlarged) containing grasshopper eggs.

This type of source has several advantages (see Sheppard, 1949). The dosage rate can be determined to an accuracy of about 2%. The long half life of the cobalt (5.3 years) makes it possible to use a given source over a period of several months with very little decrease in the dosage rate. Because the source is cylindrical and its interior is filled with polystyrene, the radiation field is quite uniform throughout the space containing the biological material. Further, low-intensity treatment can be maintained at a constant level over long periods of time inside a protective lead container occupying less than a square foot of the floor.

Ideally, all the space in the specimen vessels not occupied by the biological material should contain water. Since the complete immersion of grasshopper eggs in water affects mitoses very adversely, presumably because of the reduced exchange of oxygen and carbon dioxide between the egg and the surrounding medium, only enough moisture was placed in each vessel to keep the eggs from drying out. The pocket of air in each vessel is so small (about 0.05 cubic inch) that it should have no appreciable effect on the amount of radiation received by the eggs. The vessels were opened for a very brief period every 48 hours in the 3.4 r/hour experiment and twice daily in the 0.80 r/hour experiments during the treatment period to prevent accumulation of excessive amounts of carbon dioxide and depletion of oxygen in the vessel containing the eggs. In all experiments, irradiated and control embryos were from the same egg pods and were handled identically except for the presence or absence of the activated cobalt-aluminum cylinder in the pig.

The source was calibrated by substituting for the plug and vessels in the polystyrene bore a thimble chamber of the type designed by Darden and Sheppard (1951) and comparing the intensity of ionization with that measured when the same chamber was exposed to a radium source of known mass.

At the end of the irradiation period, which lasted for 2, 4 or 6 days, control and treated eggs were removed from the vessels and made into hanging-drop preparations by the method described previously (Carlson and Hollaender, 1944; Carlson, 1946).

Except in the final experiment, in which the numbers of middle and late prophases were also recorded, mitotic activities of the treated and control embryos were determined by counting the mid-mitotic[3] neuroblasts from the first maxillary through the first abdominal segments at 22-minute intervals. The average time required by the neuroblast to pass through mid-mitosis is about 22 minutes; therefore, the total of a number of such counts approximates the total number of cells undergoing mitosis within the period of time involved. Information on the number of counts made, the time after treatment of the initial count, the dosage rate, and the time of treatment, which differed from one experiment to another, is included with the description of the individual experiments.

## OBSERVATIONS AND INTERPRETATION

### *Dosage rate of 3.4 r/hour*

The mitotic effects of treatment for 6 days (143–145 hours) at a dosage rate of 3.4 r/hour are shown in Figure 2. The first count was made at an average of 16 minutes[4] after removal of the embryos from the source; subsequent counts were made at 22-minute intervals. Except for the second, or 38-minute, counting period, the number of mid-mitotic cells is reduced by this treatment to approximately 0.4 of normal. This is in striking contrast to what would have been obtained, if the same total dose—490 r—had been delivered in a few minutes instead of 6 days. Then the mitotic activity of the irradiated cells would have been

---

[3] This refers to cells in prometaphase, metaphase, or anaphase, *i.e.*, those between the breakdown of the nuclear membrane at the end of prophase and the loss of distinctness in the appearance of the chromosomes that marks the advent of telophase.

[4] The times after irradiation at which the initial counts were made differ from experiment to experiment, because we did not recognize the importance of an immediate first count until the early experiments had been completed.

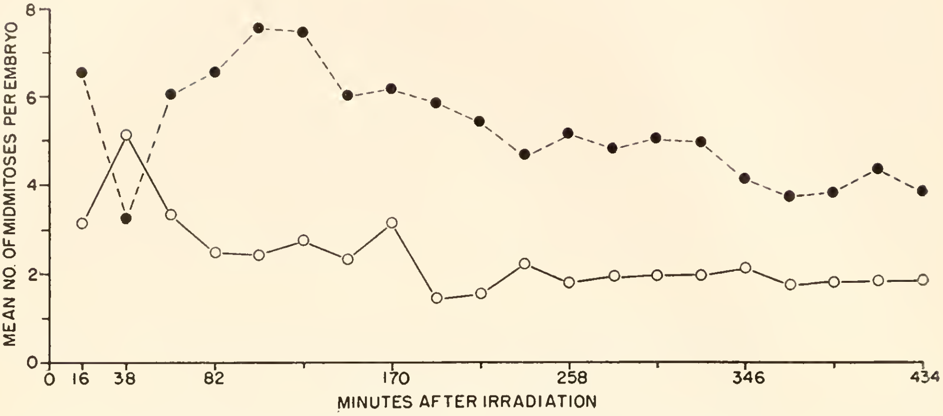


FIGURE 2. Dosage rate, 3.4 r/hour. Duration of treatment, 143–145 hours; dose, 489.6 r. ○, irradiated cells; ●, control cells

reduced to zero within an hour after treatment and would have remained at that level for about 5 hours. This demonstrates clearly the capacity of cells to undergo recovery from mitotic effects of radiation during the period of treatment.

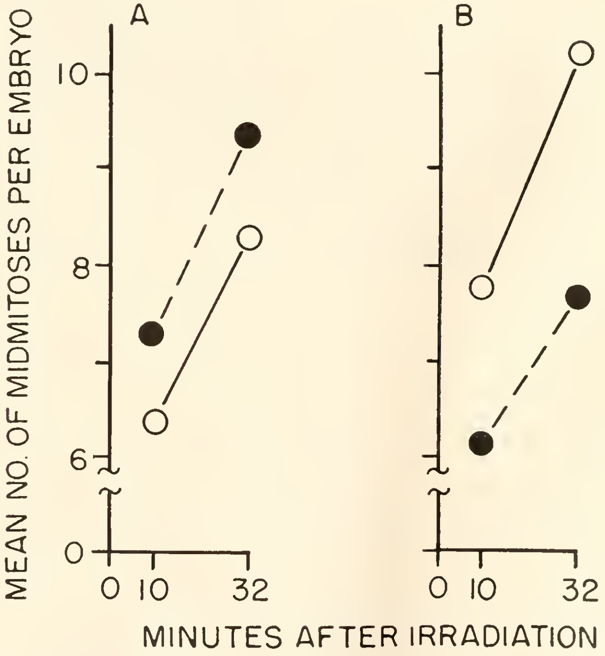


FIGURE 3. Dosage rate, 0.80 r/hour. A, duration of treatment, 44–51 hours; approximate dose, 35–41 r. B, duration of treatment, 92–99 hours; approximate dose, 74–79 r. ○, irradiated cells; ●, control cells

TABLE I

*Summary of data and biometrical analysis*

| Dose rate (r/hr.) | Duration of irradiation (hrs.) | Approximate dose (r) | No. of embryos | | No. of counting periods | Stages counted | Per cent level of significance | | Least significant difference (5% level) between treated and control embryos for any one counting period* | Standard error of a single count* | Degrees of freedom for error |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Irradiated | Control | | | Between treated and control counts averaged over all counting periods | For interaction between treatment and counting period | | | |
| 3.4 | 143–145 | 490 | 16 | 16 | 20 | Mid-mitosis | <0.1 | <0.1 | 0.46 | 0.67 | 562 |
| 0.80 | 44–51 | 35–41 | 31 | 31 | 2 | Mid-mitosis | 20 | >50 | 0.30 | 0.60 | 58 |
| | 92–99 | 74–79 | 20 | 20 | 2 | Mid-mitosis | 3.6 | 43 | 0.42 | 0.64 | 38 |
| | 144 | 115 | 11 | 11 | 16 | Mid-mitosis | >50 | <0.1 | 0.54 | 0.65 | 300 |
| | 144 | 115 | 15 | 14 | 4 | Middle prophase | 42 | >50 | 0.76 | 0.81 | 73 |
| | | | | | | Late prophase | <0.1 | 9.7 | 0.53 | 0.68 | 73 |
| | | | | | | Mid-mitosis | >50 | 18 | 0.60 | 0.89 | 77 |

* These figures should not be compared with the mean counts shown on the graphs, but with their approximate square roots. See text footnote 5.

The statistical analysis of the 3.4 r/hour data[5] summarized in Table I shows that the average numbers of mid-mitotic cells in treated and control embryos averaged for the whole counting period differ significantly at the 0.1% level of probability. Interaction is indicated by the fact that differences between treated and control embryos vary significantly at the 0.1% level from count to count.

*Dosage rate of 0.80 r/hour*

After two-day exposures at this dosage rate, mid-mitotic counts at 10 and 32 minutes after irradiation (Fig. 3A) were not significantly different from those of the control embryos, with respect to the counts averaged over all counting periods or the interaction (Table I). After four-day exposures, however, the mid-mitotic counts at both 10 and 32 minutes after irradiation were higher than in the controls (Fig. 3B). Biometrical analysis indicates a significant difference at the 3.6% level for the counts averaged over all counting periods and for the second counts alone at the 5% level, but not for the first count or for interaction (Table I).

Some preliminary 6-day exposures at this dosage rate also gave initial mid-mitotic counts that were distinctly higher than those of the control embryos. This unexpected result, it was reasoned, could have resulted from any of three factors. (1) Prolonged low-intensity radiation might have a stimulating effect on mitosis. By this we mean that a more rapid passage of cells through the stages of the mitotic cycle between the end of anaphase and the beginning of prometaphase, namely, telophase, interphase, or prophase, might throw a proportionally greater number of the cells into mid-mitosis. (2) The progress of cells through the stage being counted, namely, mid-mitosis, might have been retarded, so that the same cell was included in successive counts, thus giving a false picture of increased mitotic activity. (3) Cells might have been retarded in late prophase, accumulating there in abnormally large numbers, and then progressing simultaneously into mid-mitosis after removal from the field of radiation.

With these possibilities in mind an experiment was set up to determine whether or not this initial "stimulating" effect was the result of counting the same mid-mitotic cell twice in successive counts. Treatment was at the rate of 0.80 r/hour for 6 days (total dose, 115.2 r). Sixteen successive counts were made at 22-minute intervals beginning 22 minutes after the end of radiation. During each observation period, neuroblasts in mid-mitosis were not only counted but also the exact stage and the location of each cell were recorded. From these data it was possible to correct for all cases in which a cell was counted twice by eliminating the second record. The only counting period for which this correction averaged more than 0.3 count per embryo was the second. Corrections for that period reduced the average count from 13.3 to 11.2 per irradiated embryo and from 6.0 to 4.55 per control embryo. The corrected results are used in Figure 4.

Biometrical analysis (Table I) indicates that there is no significant difference in the mitotic activity of the treated and control embryos for the period of observation taken as a whole. Interaction, however, is significant at the 0.1% level. The means of the 22- and 44-minute counts (Fig. 4) of the treated cells are significantly higher than those of the untreated cells while the means of the 110-, 132-, and 154-minute counts of the treated cells are significantly lower than those of the control cells at the 5% level. The possibility that the higher initial number of mid-mitotic cells in the treated than in the control embryos resulting from counting the same cell twice is, therefore, eliminated.

---

[5] All analyses were based on the square root of counts, because this transformation makes variances approximately uniform.

To determine whether the high initial number of mid-mitotic cells results from simultaneous recovery and mitotic progression of an abnormally large number of cells retarded at middle and late prophase by the irradiation, an experiment was run in which the counts were made of middle prophases, late prophases and mid-mitoses. The first count was made as soon after irradiation as the material could be prepared for observation, *i.e.*, 4.5–6.0 minutes after removal from the radiation field. Three additional counts were made 22, 44, and 66 minutes after the end of radiation.

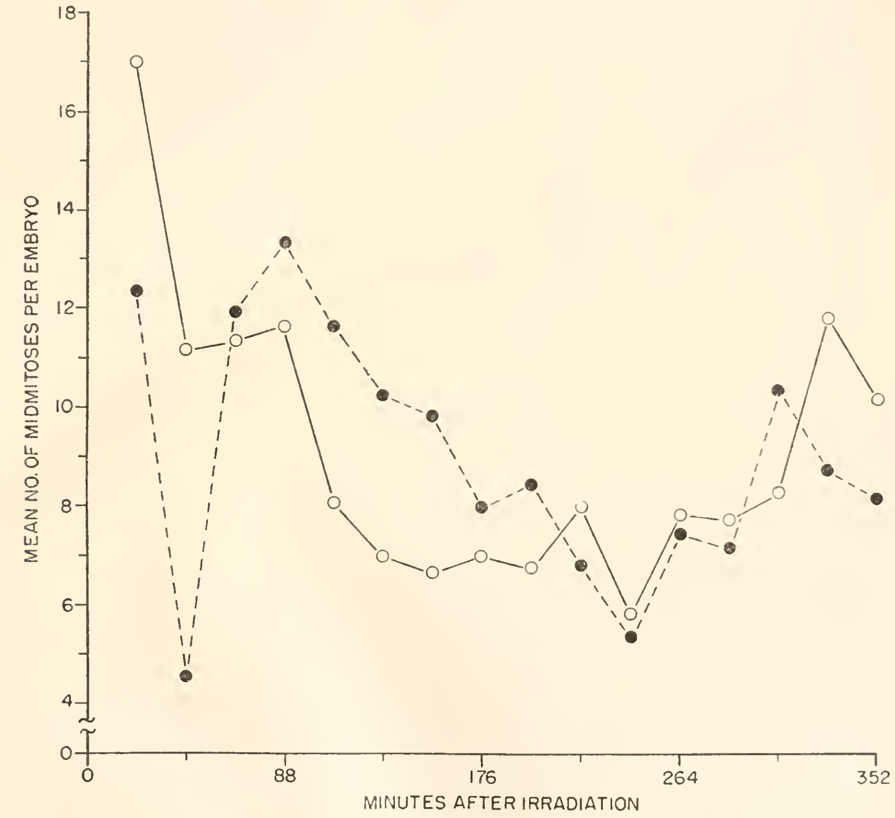


FIGURE 4. Dosage rate, 0.80 r/hour. Duration of treatment, 144 hours; dose, 115 r. ○, irradiated cells; ●, control cells

The results are shown graphically in Figure 5. The 22-minute mid-mitotic count, like that in the preceding experiment, is considerably higher than the corresponding control count. The difference is significant at the 8.2% level (Table II).

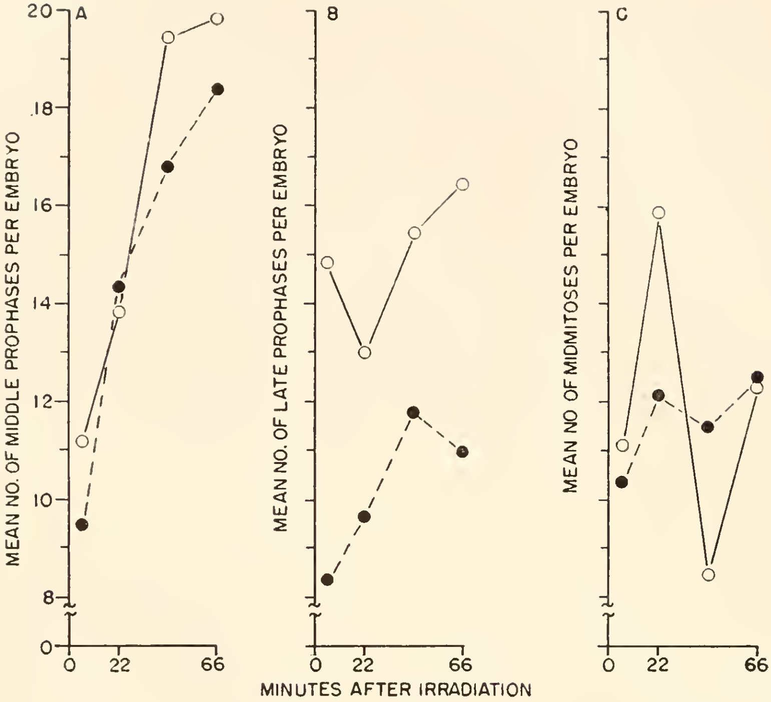


FIGURE 5. Dosage rate, 0.80 r/hour. Duration of treatment, 144 hours; approximate dose, 115 r. A, middle prophases; B, late prophases; C, mid-mitoses. ○, irradiated cells; ●, control cells

The immediate (4.5- to 6.0-), 44-, and 66-minute counts, however, are not significantly different in the irradiated and control embryos. Most of the cells in mid-mitosis at the time of the 22-minute count were in late prophase at the time of the preceding count (see mitotic time schedule, Carlson and Hollaender, 1948). Examination of the initial count of late prophase cells shows a significantly greater number of irradiated than of control late prophases at the 0.1% level. This supports the second possibility mentioned earlier that the abnormally high early

TABLE II

*Comparison of treated and control counts of middle prophases, late prophases, and mid-mitoses for four counting periods following 144 hours of irradiation at 0.80 r/hour*

| Minutes after irradiation | Per cent level of significance between irradiated and control embryos | | |
|---|---|---|---|
| | Middle prophase | Late prophase | Mid-mitosis |
| 4.5–6.0 | 55 | <0.1 | 65 |
| 22 | 88 | 5.5 | 8.2 |
| 44 | 46 | 8.5 | 21 |
| 66 | 64 | 0.5 | 87 |

mid-mitotic count results from the gradual accumulation of many cells in late prophase during the long irradiation period. That the inhibitory effect of the irradiation on late prophase cells persists in some measure even as late as 44 and 66 minutes after treatment is evidenced by the significantly greater numbers (at the 8.5 and 0.5% levels, respectively) of late prophases at these counting periods.

The irradiated middle prophases are not significantly different from the controls with respect to averages over all counting periods, interaction, or any of the four counts.

It seems worth while to point out that the mean counts (Fig. 5) in treated embryos are higher than those of the control embryos (1) for all but one of the middle prophase counting periods, (2) for all of the late prophase counting periods, and (3) for two of the four mid-mitotic counting periods. Even though only three of the higher ones are *significantly* higher at the 5.5 level, the fact that three-fourths of all of them are higher emphasizes the tendency of neuroblasts to accumulate in certain parts of the mitotic cycle when subjected to very low doses of radiation.

These results demonstrate again what has become increasingly evident over the years during which the mitotic effects of irradiation have been studied, namely, that radiation can retard but not stimulate the mitotic progress of cells. It appears that any increase in the number of cells in a given stage of mitosis soon after treatment can be interpreted to result from either retardation of mitotic progress within that stage resulting in an accumulation of cells, or entry into that stage in a brief period of time of an abnormally large number of cells that accumulated in a preceding stage as a result of mitotic retardation.

Further substantiation is also given to the transitory nature of the physicochemical radiation-induced effects that may lead ultimately to mitotic retardation. The results of earlier ultraviolet (Carlson and Hollaender, 1945) and $\gamma$-ray (Carlson, Snyder and Hollaender, 1949) studies indicated that the smaller mitotic effect of a large dose of low, as compared with high, intensity irradiation was probably due to less opportunity for interaction of primary radiation effects. In the present study the dosage rate has been so low that the chance of interaction has probably been virtually eliminated.

The authors are greatly indebted to Dr. C. W. Sheppard for designing and calibrating the $\gamma$-radiation source for special use in this project, and to Dr. A. W. Kimball for the biometrical analysis of the data.

## SUMMARY

1. Prolonged treatment of *Chortophaga* neuroblasts with low-intensity $\gamma$ radiation reduces mitotic activity much less than a comparable dose given at high intensity.

2. Treatment for 6 days at 3.4 r/hour reduced the mid-mitotic count to about 40% of normal for a period of about 7 hours following treatment. (The same dose administered in a few minutes would have reduced the mid-mitotic count to zero for about 5 hours beginning within an hour of the end of treatment.)

3. Continuous irradiation at 0.80 r/hour (1) failed to produce a significant mid-mitotic effect at the end of two days, (2) produced a significant *increase* in

the number of mid-mitoses through the second counting period at the end of four days, and (3) produced a significant *increase* in the number of mid-mitoses through the second counting period after 6 days of treatment.

4. The radiation-induced increase in the number of mid-mitoses immediately following four and six days of treatment is shown to result, not from a stimulating effect of the radiation on mitotic activity, but from the simultaneous progression into mid-mitosis of neuroblasts that have accumulated in late prophase as a result of the inhibiting effect of the radiation on this stage.

## LITERATURE CITED

Carlson, J. G., 1946. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis. *Biol. Bull.,* **90**: 109–121.

Carlson, J. G., 1950. Effects of radiation on mitosis. *J. Cell. Comp. Physiol.,* **35**, Suppl. **1**: 89–101.

Carlson, J. G., and A. Hollaender, 1944. Immediate effects of low doses of ultraviolet radiation of wavelength 2537 Å on mitosis in the grasshopper neuroblast. *J. Cell. Comp. Physiol.,* **23**: 157–169.

Carlson, J. G., and A. Hollaender, 1945. Intensity effects of ultraviolet radiation of wavelength 2537 Å on mitosis in the grasshopper neuroblast. *J. Cell. Comp. Physiol.,* **26**: 165–173.

Carlson, J. G., and A. Hollaender, 1948. Mitotic effects of ultraviolet radiation of the 2250 Å region, with special reference to the spindle and cleavage. *J. Cell. Comp. Physiol.,* **31**: 149–173.

Carlson, J. G., M. L. Snyder and A. Hollaender, 1949. Relation of gamma-ray dosage rate to mitotic effect in the grasshopper neuroblast. *J. Cell. Comp. Physiol.,* **33**: 365–372.

Darden, E. B., Jr., and C. W. Sheppard, 1951. A thimble type gamma-ray dosimeter and the measurement of the radiation from lumped and distributed type sources. USAEC Report No. ORNL–1002, Office of Technical Services, Dept. of Commerce, Washington, D. C.

Sheppard, C. W., 1949. A distributed type source for exposing biological materials to gamma rays. USAEC Report No. AECD–2566, Office of Technical Services, Dept. of Commerce, Washington, D. C.

# THE EFFECT OF VARIOUS SUSPENSION MEDIA ON THE ACTIVITY OF CHOLINESTERASE FROM FLIES

L. E. CHADWICK, J. B. LOVELL AND V. E. EGNER

*Chemical Corps Medical Laboratories, Army Chemical Center, Maryland*

The cholinesterases (ChE's) of insects merit intensive study both because of their importance for normal nervous function in these animals and on account of their consequent role in the mode of action of certain highly effective insecticides (Roeder *et al.*, 1947; Dubois and Mangun, 1947; Chadwick and Hill, 1947; Roeder, 1948; etc.). Moreover, it is to be expected that the comparative study of enzymes of this type from diverse species will speed progress toward the definition and understanding of the significant properties of ChE's generally. We have therefore investigated the acetylcholine (ACh)-splitting mechanism in the nervous tissue of houseflies.

Several properties of this system have already been reported by Metcalf and March (1949, 1950) and by Babers and Pratt (1950, 1951). In their experiments with fly-head brei, these workers adopted suspension media commonly used with vertebrate ChE's and found such solutions compatible with the insect enzyme. Our assays were begun similarly, but it soon became apparent that maximal activity of fly-head ChE is not obtained under these conditions. It has seemed worthwhile for this reason to make a systematic study of the various factors which influence the rate at which ACh is hydrolyzed by fly-head brei. The present report is concerned mainly with the effects of variation in salt concentration. Some observations with solutions of glycerol and sucrose are included.

## Material and Methods

1. *Material.* The data below refer to a strain of houseflies (*Musca domestica* L.) that was obtained early in 1948 from the testing stock of a commercial laboratory and that has been maintained in mass culture without exposure to insecticides or other toxicants. The larvae were grown at 32 degrees C. and approximately 30 per cent relative humidity (room conditions), in ground horsemeat placed between layers of sterile sawdust. The resulting pupae were blown free of sawdust and held in jars for emergence. Before mating had taken place, the adults were separated according to sex, and were kept in cages until use, with dry sugar, dry whole milk, and a source of water. Breeding stocks were housed in other cages and, in addition to the usual food and water, were supplied daily with ground horsemeat as an oviposition medium. The eggs or newly hatched larvae were then transferred to rearing jars. Under these conditions, development from egg to adult required 7 or 8 days.

2. *Preparation of tissues.* Adult flies of known age and sex were anesthetized with carbon dioxide gas. The heads were removed with fine scissors, counted into tared weighing bottles, weighed, ground, diluted to the desired concentration,

and assayed. Alternatively, batches of heads or whole flies were quick-frozen and stored indefinitely at −15 degrees C.; after thawing, they were processed at once. When necessary, tissue suspensions were held overnight or for longer periods in a refrigerator at 2 to 3 degrees C.

Breis were prepared from the heads by grinding them in a Pyrex test tube with 2 to 5 ml. of the desired suspension medium. A stainless steel pestle, rotated by a drill press at 700 r.p.m., provided a convenient and durable modification of the original all-glass Potter-Elvehjem homogenizer. Small samples, containing from 1 to 20 heads, were ground at room temperature for 30 to 35 seconds. Larger samples, up to 500 heads, were ground for longer periods, in a test tube surrounded by cracked ice. No abrasive was added.

3. *Method of assay.* Production of acid from ACh.Br was measured as proposed by Glick (1937), by means of titration with standard alkali at "constant" pH, using the Beckman Model G meter as a null instrument. The following conditions were adopted: tissue concentration, one head per ml.; final volume of sample, 20 ml.; reaction vessel, 50 ml. beaker; temperature, 25.0 degrees C.; pH, 8.0; rate of addition of 0.1 N NaOH during assay, 0.01 or 0.02 ml. as often as required to keep pH from dropping below 8.0; duration of measurement, approximately 15 minutes; beaker contents agitated by hand every 15 to 30 seconds; initial concentration of ACh.Br in assay mixture, 0.015 *M*.

Concentrated breis, containing 10 ground heads per ml., were made up in water, and two-ml. aliquots were diluted with stock reagent and water so as to provide a tissue concentration of one head per ml. in the desired strength of reagent. The reagent and sample containers were kept partially immersed on a shelf in the constant temperature bath. After addition of ACh.Br to the sample, pH was rapidly adjusted to slightly above 8.0 with 0.1 N NaOH or HCl. The pH was then allowed to fall to 8.0, at which time a stopwatch was started and 0.01 or 0.02 ml. of 0.1 N NaOH added by pipette. This raised pH to a value between 8.0 and (in extreme cases) 8.5; it was again allowed to fall, with stirring, and when 8.0 had been reached again the time was recorded to the nearest second and another 0.01 or 0.02 ml. of 0.1 N NaOH added. This routine was repeated as often as necessary for approximately 15 minutes. The rate of production of acid was then calculated from the elapsed time and the total volume of alkali used.

The reproducibility of the technique was measured by determining the activity of 20 aliquots, equivalent to 20 heads each, from a single sample of brei, which was suspended in a buffered solution of the following composition: NaCl, 26.30 gm.; $KH_2PO_4$, 3.85 gm.; NaOH, 1.00 gm.; $H_2O$, to one liter; approximate normality with respect to cations, 0.5; pH adjusted to 8.0. The average ChE activity of this preparation, after correction for acid produced from other sources, was 5.15 ± 0.23 micromoles ACh.Br hydrolyzed per head per hour. Thus, the coefficient of variability amounted to ± 4.5 per cent. Repetitions in unbuffered 0.5 N $MgCl_2$ and 0.5 N NaCl with 16 aliquots each gave a coefficient of ± 2.8 per cent in both cases. Variation was of the same order when 20-head aliquots were individually prepared. Such a test in buffer with 20 samples from a batch of male flies 5 to 6 days old yielded a coefficient of variability of ± 3.8 per cent.

4. *Reagents.* With the exception of glycerin, which was of USP quality, 98 per cent pure, reagents were of CP grade. Stock solutions were prepared with

de-ionized water. The concentrations of the chlorides were checked by titration with 0.1 N $AgNO_3$. Alkali employed for titrating acid that was produced by the samples was rechecked against standard 0.1 N HCl at intervals. The loss of alkalinity amounted to less than 2 per cent in several months. ACh.Br, recrystallized from the Paragon or Matheson product, was made up fresh daily at 0.15 *M* concentration in de-ionized water.

5. *Treatment of data.* The rates reported represent enzymic hydrolysis, the

TABLE I

*ChE activity of fly-head suspensions as a function of concentration of single salts, glycerol or sucrose*

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NaCl | | | | | | | | | | | | |
| Normality | 0 | 0.0001 | 0.001 | 0.01 | 0.06 | 0.12 | 0.24 | 0.50 | 1.08 | 1.62 | 2.16 | 3.24 |
| Activity in per cent* | | | | | | | | | | | | |
| Average | 100 | 105 | 107 | 122 | 188 | 196 | 222 | 242 | 238 | 213 | 190 | 121 |
| Maximum | — | 106 | 110 | 127 | 216 | 209 | 237 | 267 | 255 | 235 | 201 | 128 |
| Minimum | — | 104 | 105 | 118 | 177 | 174 | 200 | 200 | 221 | 192 | 182 | 114 |
| Number of samples | 7 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Correction** | −0.56 | −0.56 | −0.56 | −0.55 | −0.52 | −0.46 | −0.50 | −0.33 | −0.26 | −0.25 | −0.24 | −0.22 |
| KCl | | | | | | | | | | | | |
| Normality | 0 | 0.036 | 0.072 | 0.15 | 0.27 | 0.54 | 0.81 | 1.08 | 1.62 | | | |
| Activity in per cent* | | | | | | | | | | | | |
| Average | 100 | 168 | 192 | 218 | 242 | 248 | 247 | 231 | 204 | | | |
| Maximum | — | 183 | 212 | 241 | 263 | 264 | 262 | 249 | 218 | | | |
| Minimum | — | 150 | 171 | 162 | 209 | 221 | 230 | 217 | 170 | | | |
| Number of samples | 7 | 5 | 5 | 5 | 5 | 6 | 5 | 5 | 7 | | | |
| Correction** | −0.56 | −0.41 | −0.41 | −0.37 | −0.33 | −0.31 | −0.27 | −0.26 | −0.24 | | | |
| $MgCl_2$ | | | | | | | | | | | | |
| Normality | 0 | 0.0001 | 0.001 | 0.01 | 0.058 | 0.115 | 0.23 | 0.46 | 0.92 | 1.84 | 3.68 | |
| Activity in per cent* | | | | | | | | | | | | |
| Average | 100 | 102 | 118 | 162 | 241 | 255 | 257 | 271 | 268 | 217 | 38 | |
| Maximum | — | 106 | 121 | 164 | 255 | 268 | 279 | 296 | 277 | 235 | 47 | |
| Minimum | — | 96 | 114 | 157 | 225 | 230 | 225 | 252 | 257 | 194 | 30 | |
| Number of samples | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | |
| Correction** | −0.56 | −0.56 | −0.55 | −0.47 | −0.29 | −0.29 | −0.32 | −0.34 | −0.46 | −0.70 | −1.19 | |
| $CaCl_2$ | | | | | | | | | | | | |
| Normality | 0 | 0.051 | 0.102 | 0.20 | 0.41 | 0.81 | 1.63 | 3.26 | | | | |
| Activity in per cent* | | | | | | | | | | | | |
| Average | 100 | 187 | 197 | 229 | 242 | 199 | 101 | nil | | | | |
| Maximum | — | 228 | 241 | 250 | 267 | 219 | 128 | −1 | | | | |
| Minimum | — | 198 | 207 | 190 | 212 | 190 | 112 | −16 | | | | |
| Number of samples | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | | | | |
| Correction** | −0.56 | −0.39 | −0.41 | −0.42 | −0.46 | −0.54 | −0.71 | −1.22 | | | | |
| $NaNO_3$ | | | | | | | | | | | | |
| Normality | 0 | 0.06 | 0.12 | 0.24 | 0.50 | 1.08 | 1.62 | 2.16 | 3.24 | | | |
| Activity in per cent* | | | | | | | | | | | | |
| Average | 100 | 173 | 194 | 200 | 207 | 190 | 170 | 148 | 113 | | | |
| Maximum | — | 176 | 198 | 204 | 216 | 194 | 174 | 151 | 123 | | | |
| Minimum | — | 163 | 181 | 194 | 200 | 179 | 164 | 143 | 104 | | | |
| Number of samples | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | | | |
| Correction** | −0.56 | −0.38 | −0.36 | −0.35 | −0.32 | −0.32 | −0.29 | −0.25 | −0.24 | | | |
| Glycerol | | | | | | | | | | | | |
| Molarity | | 0 | 0.125 | 0.25 | 0.50 | 1.00 | 2.00 | 4.00 | | | | |
| Average activity in per cent* | | 100 | 99 | 97 | 93 | 88 | 70 | 46 | | | | |
| Number of samples | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | | | |
| Correction** | | −0.56 | — | — | — | −0.51 | −0.54 | −0.50 | | | | |
| | | | | (used −0.56 for all concentrations) | | | | | | | | |
| Sucrose | | | | | | | | | | | | |
| Molarity | | 0 | 0.03125 | 0.0625 | 0.125 | 0.25 | 0.45 | 0.90 | 1.35 | | | |
| Average activity in per cent* | | 100 | 104 | 102 | 97 | 91 | 89 | 73 | 59 | | | |
| Number of samples | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | | |
| Correction** | | −0.56 | — | — | — | — | −0.47 | — | −0.64 | | | |
| | | | | (used −0.56 for all concentrations) | | | | | | | | |

* Activity expressed in terms of that of the same breis in water = 100 per cent, as follows: for NaCl, 2.70 micromoles ACh·Br hydrolyzed per head per hr.; for KCl, 2.67; for $MgCl_2$, 2.23; for $CaCl_2$, 2.38; for $NaNO_3$, 2.45; for glycerol and sucrose, 2.44. Tissue concentration, one head per ml.

** Corrections (for acid produced from sources other than enzymic hydrolysis of ACh·Br) are in microequivalents per ml. per hr., and were applied to the raw data before calculation of per cent activity. All runs at 25.0 degrees C.; pH, 8.0.

total acid production having been corrected by subtraction of the fraction contributed by processes other than enzymic breakdown of substrate in the particular medium concerned. These correction values were determined by titration of 20-ml. aliquots that contained the usual concentrations of tissue and ACh.Br, but whose ChE activity had been destroyed by incubation of the stock brei overnight or longer with $1 \times 10^{-5}$ $M$ di-isopropyl fluorophosphate (DFP).

Net measurements of enzymic activity were converted into micromoles of ACh.Br hydrolyzed per mg. fresh weight *and* per head per hour. Our results are reported only on the latter basis. The head and body weight of flies varies with age, among other factors, and there are changes in proportion as well as amount of water and other constituents. Moreover, a large and likewise varying fraction of the total weight consists of cuticle, which is presumably free of ChE activity. In contrast with these variations, ChE activity per head remains relatively constant, at a somewhat different level in each sex, after the first day or two of adult life. On this account, it has been advantageous to make comparisons on a per-head basis rather than with reference to fresh or dry weight, or protein or nitrogen content. The average fresh weight of a head in most of our samples has been between 1.2 and 2.9 mg. Dry weight in different batches ranged from 0.33 to 0.65 mg., and from 22 to 36 per cent of the total.

## RESULTS

Table I shows ChE activities of fly-head suspensions as affected by various concentrations of single salts, glycerol or sucrose. Comparison has been simplified by expressing the average rates as percentages of the activity found for the same breis in the absence of added reagent. Such treatment of the data is considered proper because the degree of activation or depression observed with a given concentration of reagent, although somewhat variable from one suspension to another, was found to be independent of the absolute level of activity of the preparation. The outcome of a fresh comparison of the salts at nearly optimal concentrations, in which all runs were made with aliquots of the same stock brei, is shown in Table II. Not listed is a single test with 0.5 N $(NH_4)_2SO_4$, in which ChE activity was about twice that of the same suspension in water. This salt solution was too strongly buffered to permit satisfactory measurement of the necessary correction. Results of a few other experiments are cited in the discussion.

TABLE II

*Comparison of ChE activity of fly-head suspensions in 0.5 N solutions of single salts*

| Salt | $NaCl$ | $KCl$ | $MgCl_2$ | $CaCl_2$ |
|---|---|---|---|---|
| Activity in per cent* | | | | |
| Average | 212 | 201 | 229 | 208 |
| Range | 204–217 | 194–212 | 213–254 | 197–228 |
| Number of observations | 5 | 5 | 5 | 5 |

* Activity expressed in terms of that in water (1.78 micromoles ACh·Br hydrolyzed per head per hour) = 100 per cent. All determinations made on aliquots of the same brei, at 25.0 degrees C., pH 8.0.

## DISCUSSION

1. *Non-enzymic hydrolysis.* Under our conditions of measurement, at pH 8.0 and 25.0 degrees C., 0.015 *M* ACh.Br in buffered solution or in the higher concentrations of monovalent salts produced 0.22 to 0.24 microequivalents of acid per ml. per hr., in either the presence or absence of the usual concentration (one head per ml.) of DFP-inhibited tissue. These results compare reasonably well with values approximated by interpolation from the data of Augustinsson (1948) for non-enzymic hydrolysis of ACh.Cl and ACh.Br in bicarbonate buffer.

As may be seen from Table I, we observed and have used for correction considerably higher values in many of our experiments.

The observations with the salt solutions fall into two distinct groups. With the monovalent compounds, that portion of the total acid production that was not inhibited by DFP decreased more or less exponentially as salt concentration was increased. With the chlorides of divalent metals, on the contrary, an initial drop in acid production at low salt concentrations was followed by an increase that was related linearly to the concentration of salt.

In both cases, apparently, the higher rates at very low concentrations were the product of a technical shortcoming; namely, that addition of alkali during titration raised the pH of the medium temporarily and thereby accelerated the hydrolysis of ACh.Br. This effect was naturally more marked the smaller the buffering capacity of the medium; it was therefore greater in the more dilute solutions, where addition of 0.01 ml. of 0.1 N NaOH to a 20-ml. sample raised pH from 8.0 to 8.5 or occasionally even higher. Lower correction values were obtained with these dilute solutions when alkali was added in 0.0025 ml. amounts, but these results, of course, were not applicable to our experimental conditions.

The increase in acid production observed when concentration of $MgCl_2$ or $CaCl_2$ was above 0.1 N probably reflects a true increase in the rate of hydrolysis of ACh.Br. At pH 8.0 such solutions are quite strongly buffered, so that changes in pH on addition of alkali during titration become very small and cannot be held responsible for the effects noted. In the absence of substrate, tissue in 4.0 N $MgCl_2$ produced about 0.1 microequivalent of acid per ml. per hr. Ten times as much acid was released when substrate was present and tissue omitted. The increased hydrolysis in strong solutions of $MgCl_2$ is evidently not dependent on the presence of tissue, and it may be mentioned in passing that DFP-inhibited enzyme is not reactivated at all by incubation with either monovalent or divalent salts at 4.0 N concentration, even after several days. Other tests with 0.015 *M* ACh.Br in 4.0 N $MgCl_2$, where the loss of ester over periods of one to three hours in the presence of DFP-inhibited enzyme was determined chemically by the method of Hestrin (1949), indicated a hydrolysis rate of about 1.1 micromoles ACh.Br per ml. per hr. at 25.0 degrees C. and pH 8.0. These results may be compared with the correction value of 1.19 shown in Table I for 3.68 N $MgCl_2$.

The saddle-backed curve of correction values observed with the divalent salts seems, then, to owe its shape to a combination of two factors: (1) an inadequacy of procedure that resulted in slightly higher average pH during titration in very weak solutions; and (2) an opposing tendency toward acceleration of hydrolysis of ACh.Br as the concentration of divalent ions was increased. The latter process seems to be related linearly to salt concentration. We have assumed that similar factors are concerned in the results with $CaCl_2$, although our attempt to analyze the situation has been confined to solutions containing $MgCl_2$.

The correction values obtained with glycerol and sucrose at all concentrations tested differed little from those observed in plain water, except for some indications of a trend toward higher rates of acid production at the higher concentrations of these compounds. This trend was more conspicuous in a few tests where substrate concentration was 0.06 *M* instead of the usual 0.015 *M*.

In concluding this section of the discussion, we would like to emphasize that, in some contrast with non-enzymic hydrolysis, the rate of breakdown of ACh.Br by ChE is but slightly affected by pH changes of as much as 0.5 unit in the neighborhood of pH 8.0, since activity at pH 8.5 is only 104 per cent of activity at 8.0. Use of correction values that were determined under conditions as nearly as possible identical with those prevailing during measurement of total activity therefore yields net results which should approximate closely the true rates of reaction between enzyme and substrate under the stated conditions. It is well to recognize that in certain circumstances the correction values may include acid production from a variety of sources other than what is usually thought of as non-enzymic hydrolysis, and that the rate of non-enzymic hydrolysis may be altered

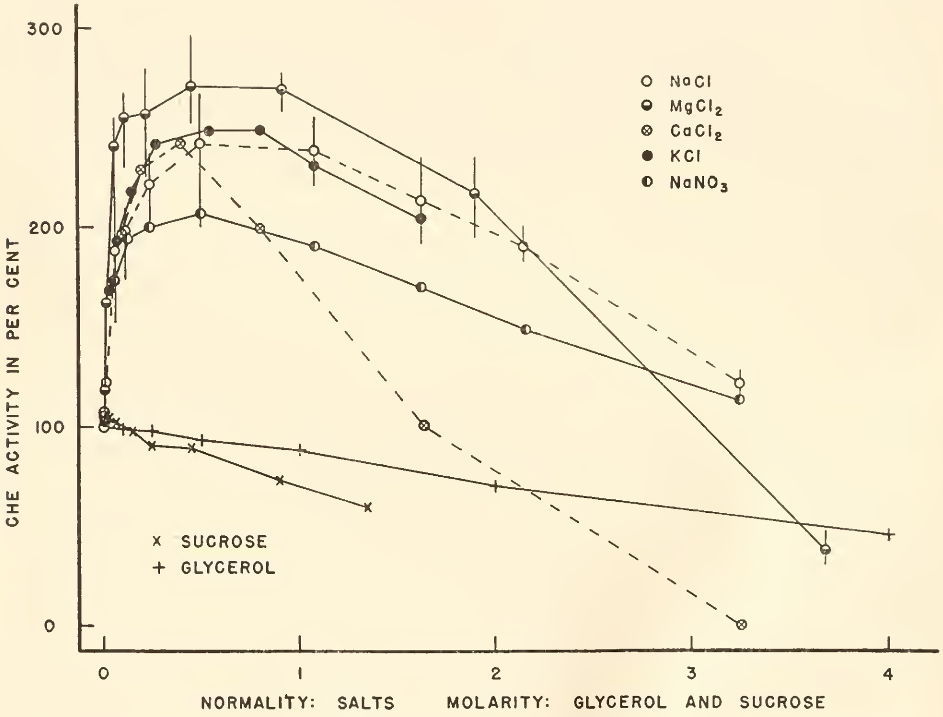


FIGURE 1. Relationship between ChE activity of fly heads and concentration of salt, glycerol or sucrose. Vertical bars indicate limits of variation encountered with NaCl and $MgCl_2$ solutions. For the range of variation with other salts, see Table I.

several-fold as a result of details of technique or variation in the composition of the medium, even though pH and temperature are held within narrow limits.

2. *Variations in ChE activity.* From Tables I and II it is evident that added salt up to 0.5 to 1.0 N had a non-specific activating effect on fly-head ChE, and that this effect was reversed at still higher concentrations. With all the salts tested, the activity-concentration curves were quite similar when concentration was expressed in terms of normality, but the correspondence was less good when comparison was based on molarity or ionic strength. The maximal rate of enzymic hydrolysis was observed in all instances at salt concentrations ca. 0.5 N, and was some two to three times the value measured with water suspensions (Fig. 1).

Relationships of this type have not been reported for other ChE's, although certain scattered data are suggestive (*e.g.*, Alles and Hawes, 1940, with human erythrocytes). The somewhat discordant literature on the subject has been summarized by Augustinsson (1948), who ascribed the disagreements to the use of enzymes from varied sources. Undoubtedly, differences do exist in the response of various ChE's to the several ions, and for this reason it is perhaps surprising that Jandorf (1950), in studies of the esterase activity of chymotrypsin, obtained results strikingly similar to ours. However, in Jandorf's experiments activity seemed to be correlated with the ionic strength of the solutions.[1]

With the fly-head preparations, none of the ions tested appears to have the role of a necessary coenzyme. Aqueous suspensions, even as dilute as 0.1 head per ml., regularly yielded $Q_{ChE's}$ (at 25.0 degrees C.) of 15 to 25 mg. ACh split per 100 mg. fresh weight of tissue per hour. During dialysis against distilled or deionized water, loss of activity was slow, and not restored by added salts. Addition of any of the salts to these dialyzed preparations caused only the proportional change in activity already familiar from the studies with freshly prepared heads. Lack of specificity was likewise apparent in experiments with salt mixtures; for example:

| Composition of medium: | water | 0.5 N NaCl | 0.5 N $MgCl_2$ | 0.25 N NaCl and 0.25 N $MgCl_2$ |
|---|---|---|---|---|
| Activity in per cent: | 100 | 258 | 275 | 263 |

In view of these various observations and the data in Tables I and II, it is unlikely that any ions present in the heads in a diffusible state (other than $H^+$ and $OH^-$) have an essential role in the activity of fly-head ChE. Nevertheless, slight differences in degree of activation were observed with some of the salts at equivalent concentrations. Thus, in both Tables I and II, as in the comparison cited in the preceding paragraph, it is apparent that the highest activity was found when $MgCl_2$ was present. The statistical significance of this difference was determined in an additional experiment. A stock brei was prepared, and 5 aliquots were run in water, 16 in 0.5 N NaCl, and another 16 in 0.5 N $MgCl_2$. The average (net) activity in water, taken as 100 per cent, was 2.32 micromoles ACh.Br hydrolyzed per head per hr. (range, 2.29 to 2.35). In 0.5 N $MgCl_2$, the average ± the standard error was 256 ± 1.8 per cent; and in 0.5 N NaCl, 232 ± 1.6 per cent. The difference is 24 ± 2.5 per cent, or more than 9 times its standard error. Thus, there is little room for doubt that $MgCl_2$ does have a slightly greater potency for activation than does NaCl, and it is likely that further investigation would disclose equally significant though small differences among some of the other salts. It is also clear from our data that, after the peak at about 0.5 N is reached, the ChE activity of suspensions containing $CaCl_2$ declines much more rapidly than it does in other salt solutions. Some specificity in the effects of the different cations has therefore been demonstrated, but this question will need re-investigation when purified enzyme is available.

---

[1] Results similar in some respects to ours have been reported recently by C. van der Meer. 1953. Effect of calcium chloride on choline esterase. *Nature*, 171: 78–79.

The mechanism of the non-specific activation seen with all the six salts tested is not known. Enzyme solubility can hardly be concerned, since filtrates or supernatants from aqueous suspensions of ground heads were found to contain well over 90 per cent of the total activity and responded to addition of salts exactly as did the crude brei. Besides, salt concentrations above 1.0 N depressed activity without necessarily precipitating or destroying equivalent amounts of enzyme. Thus, for instance, when a suspension was made 5.5 N with NaCl and centrifuged, 86 per cent of the original activity was recovered in the supernatant. (Comparison was made in 0.5 N NaCl.)

The depression of activity seen with the higher concentrations of salts is, then, very largely reversible, as is the activation noted at lower concentrations. Possibly the reduction in reaction rate at high concentrations could be explained on the basis that the dense cloud of cations around the enzyme tends to block electronegative sites that are concerned in formation of an enzyme-substrate complex, in accordance with the theory advanced by Wilson and Bergmann (1950a, 1950b). But it is not easy to relate the progressive activation by salts in concentrations up to 0.5 N to this concept, although an indication that the observed changes in activity may involve some alteration of the enzyme itself is provided by the observation that a 4-fold increase in substrate concentration failed to accelerate the rate of reaction in 3.12 N NaCl. This fact argues against the interpretation that the reduced activity observed at this salt concentration was the result of direct interference with access of substrate.

The question may be raised, in view of the analysis by Wilson and Bergmann (1950a), whether changes in the ionic composition of the suspension medium may not cause shifts in the pS optimum and thus complicate activity comparisons of the sort we have made. Experiments undertaken to settle this point by determining the substrate optimum in three media, *viz.*, de-ionized water, 0.5 N NaCl and 3.0 N NaCl, gave somewhat inconsistent results on replication. In each instance, 5 runs were made at each substrate concentration in the series 0.1, 0.03, 0.01, 0.003 and 0.001 *M*. Despite the variation encountered, a trend toward increase in substrate optimum with increasing concentration of salt may be discerned in the averages (Table III) and we would estimate the respective optima provisionally as follows: water, 0.0016 *M*; 0.5 N NaCl, 0.0025 *M*; 3.0 N NaCl, 0.005 *M*. The corresponding enzymic activities, interpolated from the average data obtained at the concentrations actually tested, were in the ratio of 100:242:129. These are so close to the ratios observed with the same salt concentrations in Table I, where all runs were made with ACh.Br 0.015 *M*, as to leave no doubt that any shift in $pS_{opt.}$ with variation in salt concentration has played a negligible role in the comparisons with which this study is primarily concerned.

Glycerol was tested in order to provide a basis for future comparison of our observations with those of Babers and Pratt (1950), who used it in 30 per cent strength for preparation of stock breis. Since, in contrast to our experience with salts, we found that ChE activity was depressed progressively as concentration of glycerol was increased, we added a series of tests with sucrose, as another example of a non-electrolyte. Here, too, activity appeared to bear an inverse relationship to concentration, with the distinction that the slope of about −31 per cent per mole is appreciably steeper than the slope found with glycerol (about −14 per cent per mole).

Table III

*Variation in activity of fly-head ChE in various media as a function of substrate concentration*

| Molar concentration of ACh·Br ........ | 0.001 | 0.003 | 0.01 | 0.03 | 0.10 |
|---|---|---|---|---|---|
| | Average enzymic activity in micromoles per head per hour | | | | |
| Suspension medium | | | | | |
| Water | 2.46 | 2.32 | 1.89 | 1.99 | 2.12 |
| 0.5 N NaCl | 4.82 | 4.91 | 4.40 | 3.51 | 2.49 |
| 3.0 N NaCl | 2.96 | 3.22 | 2.99 | 2.86 | 2.37 |

Each series of observations is the mean of 5 replications.
All runs at 25.0 degrees C., pH 8.0.

Inasmuch as there seemed to be little likelihood that either of these compounds was acting as a chemical inhibitor, we were inclined to seek some physical mechanism, such as interference with diffusion of substrate toward or reaction products away from the active sites on the enzyme, as an explanation of the changes in activity observed. In such a case, the rate of hydrolysis might be expected, as a first approximation, to be related inversely to the viscosity of the medium. Unfortunately for this hypothesis, the viscosity of aqueous solutions of glycerol or sucrose does not vary linearly with concentration, whereas the depression of ChE activity did. Moreover, activity was depressed to about the same degree in 1.35 *M* sucrose, with a viscosity of about 5 centipoises, as in 4.0 *M* glycerol, whose viscosity is only some 2.5 centipoises. And in addition, as with the concentrated salt solutions, raising substrate concentration to 0.06 *M* did not yield greater activity in the presence of 4.0 *M* glycerol or 1.35 *M* sucrose; in fact, the rate of hydrolysis was reduced some 10 to 15 per cent in comparison with that obtained with 0.015 *M* ACh.Br, a result similar to what was seen with suspensions in plain water. Kodera (1928) also concluded that viscosity changes were not responsible for the inhibitory effect of gum arabic and soluble starch on the ChE's of human serum and red cells; and suggested that the test materials might have been adsorbed to the enzyme surface and thus have masked the active sites. His data, like ours with glycerol and sucrose, resemble the results with salt solutions to the extent that they indicate that the observed changes in activity are due to some alteration of the enzyme. This could consist in blocking of the active sites as a consequence of adsorption or of some more fundamental alteration in the properties of the enzyme molecule.

Although further elucidation of the nature of these changes would be most desirable, it does not appear possible on the basis of present information. One may nevertheless draw the practical conclusion that, for experiments where maximal activity of fly-head ChE is desired, it will be well to have some salt present at about 0.5 N concentration, and to avoid the presence of compounds such as glycerol and sucrose.

The authors wish to acknowledge the kindness of Dr. Wm. E. Dove in supplying the pupae from which our fly culture was started.

## SUMMARY

1. The cholinesterase (ChE) activity of fly-head suspensions was measured titrimetrically at 25.0 degrees C. and pH 8.0 with acetylcholine bromide 0.015 *M* as substrate, as a function of the concentration of various single salts, glycerol, or sucrose. The species tested was *Musca domestica* L.

2. The salts, NaCl, KCl, $MgCl_2$, $CaCl_2$, $NaNO_3$, $(NH_4)_2SO_4$, at concentrations up to 0.5 to 1.0 N, had a non-specific activating effect. At still higher concentrations, activity was depressed progressively below the maximum, which was two to three times the value observed with water suspensions.

3. None of the ions tested was found essential to the activity of fly-head ChE. $MgCl_2$ was slightly but significantly more potent as an activator than NaCl.

4. Both glycerol and sucrose were depressant at all concentrations tested. The relationship was linear with concentration. For glycerol, the slope was about − 14 per cent per mole and for sucrose, about − 31 per cent per mole.

5. The depression of activity in the presence of higher concentrations of salts, glycerol or sucrose was not relieved by a four-fold increase in substrate concentration.

6. It is inferred that the changes in activity observed reflect alterations of an unknown nature in the properties of the enzyme, rather than direct interference with access of substrate to the active sites.

## LITERATURE CITED

Alles, G. A., and R. C. Hawes, 1940. Cholinesterases in the blood of man. *J. Biol. Chem.*, **133**: 375–390.

Augustinsson, K.-B., 1948. Cholinesterase—a study in comparative enzymology. *Acta Physiol. Scand.*, **15**: Suppl. 52: 1–182.

Babers, F. H., and J. J. Pratt, Jr., 1950. Studies on the resistance of insects to insecticides. I. Cholinesterase in flies (*Musca domestica* L.) resistant to DDT. *Physiol. Zool.*, **23**: 58–63.

Babers, F. H., and J. J. Pratt, Jr., 1951. A comparison of the cholinesterase in the heads of the house fly, the cockroach and the honey bee. *Physiol. Zool.*, **24**: 127–131.

Chadwick, L. E., and D. L. Hill, 1947. Inhibition of cholinesterase by di-isopropyl fluorophosphate, physostigmine and hexaethyl tetraphosphate in the roach. *J. Neurophysiol.*, **10**: 235–246.

DuBois, K. P., and G. H. Mangun, 1947. Effect of hexaethyl tetraphosphate on choline esterase *in vitro* and *in vivo*. *Proc. Soc. Exp. Biol. Med.*, **64**: 137–141.

Glick, D., 1937. LXXII. Properties of choline esterase in human serum. *Biochem. J.*, **31**: 521–525.

Hestrin, S., 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. Biol. Chem.*, **180**: 249–261.

Jandorf, B. J., 1950. Potentiation of esterase activity of chymotrypsin by electrolytes. *Fed. Proc.*, **9**: 186.

Kodera, Y., 1928. Über das Schicksal des Acetylcholins im Blute. V. Mitteilung. Der Einfluss von Gummi arabicum und Stärke auf den Spaltungsvorgang. *Pflügers Arch.*, **219**: 686-693.

Metcalf, R. L., and R. B. March, 1949. Studies of the mode of action of parathion and its derivatives and their toxicity to insects. *J. Econ. Ent.*, **42**: 721–728.

Metcalf, R. L., and R. B. March, 1950. Properties of acetylcholine esterases from the bee, the fly and the mouse and their relation to insecticide action. *J. Econ. Ent.*, **43**: 670–677.

Roeder, K. D., 1948. V. The effect of anticholinesterases and related substances on nervous activity in the cockroach. *Bull. Johns Hopkins Hosp.*, **83**.: 587–599.

Roeder, K. D., N. K. Kennedy and E. A. Samson, 1947. Synaptic conduction to giant fibers of the cockroach and the action of anticholinesterases. *J. Neurophysiol.*, **10**: 1–10.

Wilson, I. B., and F. Bergmann, 1950a. Studies on cholinesterase. VII. The active surface of acetylcholine esterase derived from effects of pH on inhibitors. *J. Biol. Chem.*, **185**: 479–489.

Wilson, I. B., and F. Bergmann, 1950b. Acetylcholinesterase. VIII. Dissociation constants of the active groups. *J. Biol. Chem.*, **186**: 683–692.

# ON FOOD AND FEEDING OF LARVAE OF THE AMERICAN OYSTER, C. VIRGINICA

HARRY C. DAVIS

*U. S. Fish and Wildlife Service, Milford, Connecticut*

Feeding experiments have shown that larvae of the American oyster, *Crassostrea virginica,* are limited as to the types of food they can utilize, by factors other than the size of the micro-organism or particle. We have previously reported four species of marine bacteria and three species of flagellates that had no effect on the rate of growth of oyster larvae (Davis, 1950a). In one experiment, for example, in cultures that received sulfur bacteria as food, the larvae averaged only 94.05 $\mu$ in length at 14 days, a size not significantly different from that of larvae in unfed control cultures. Moreover, it was an increase of only about 20 $\mu$ over the initial size of early straight-hinged larvae which usually measure about 74–76 $\mu$ in length. In a pair of parallel cultures fed mixed phytoplankton composed chiefly of Chlorella, the larvae averaged 140.75 $\mu$ in length by the 14th day or had gained about 65 $\mu$ over their initial size.

The same series of experiments also showed that marine detritus, which we had collected from several sources, was not utilized by the larvae, and that organic media added to the larval cultures caused no increase in the rate of growth of the larvae.

On the basis of the above experiments it was reported (Davis, 1950a) that our mass culture of green phytoplankton consisting chiefly of Chlorella, which we shall refer to as "mixed Chlorella," was the best food for oyster larvae then available. It did not consistently induce a good rate of growth when fed to larvae during their earlier stages, but once the larvae had attained a size of approximately 125 $\mu$ they appeared to utilize the "mixed Chlorella" quite readily.

In the feeding experiments on oyster larvae reported in the following pages, we have tested nine additional species of marine bacteria, isolated from the mud of Milford Harbor by Dr. Burkholder of Yale University; six more species of marine flagellates, including some of the same varieties used by Bruce, Knight and Parke (1940), obtained from Dr. Russell of the Plymouth Laboratory, England; and a bacteria-free culture of *Chlorella* sp. isolated from our mixed phytoplankton culture by Dr. Ralph Lewin of Yale University. These feeding experiments were conducted during the winter and early spring using oysters brought to spawning condition by the method described by Loosanoff (1945) and by Loosanoff and Davis (1952).

To insure uniformity in size, quality, and number of larvae in the different culture jars of a series, at the beginning of an experiment, and except for foods, to provide as nearly equal treatment of all cultures as possible, the following procedure has been made standard practice.

To obtain fertilized eggs, the conditioned oysters were placed in spawning dishes, filled with sea water that had been filtered through cotton to remove debris and most planktonic forms, and spawning was induced by adding small quantities of sperm suspension and raising the temperature to about 30.0° C. The eggs of *C. virginica,* because of their small size, cannot readily be separated from excess sperm, as can those of *Venus mercenaria* (Loosanoff and Davis, 1950). The spawning females were, therefore, separated from the males and placed in spawning dishes containing only a slight excess of sperm.

The fertilized eggs were collected in a tall narrow jar by screening the contents of the spawning dishes through stainless steel screens having 100 meshes per inch. This screen allowed the fertilized eggs to pass through unharmed but retained larger debris and feces expelled by the oysters during spawning. Since we wished to have 5000 larvae per liter in our experimental cultures, the egg suspension in the tall narrow jar was thoroughly stirred with a perforated plastic plunger to insure uniform distribution of the eggs and a sample was withdrawn and the number of fertilized eggs per ml. determined.

Only rarely did 100 per cent of the fertilized eggs develop into normal straight-hinged veliger larvae, but if less than 50 per cent developed normally, we discarded the larvae. It became our practice, therefore, to introduce enough fertilized eggs into each culture jar to give approximately twice the desired number of larvae if all the eggs should develop normally, *i.e.*, about 10,000 fertilized eggs per liter. The 20-liter earthenware culture jars were then filled with cotton-filtered sea water and the eggs permitted to develop for 48 hours with no supplemental feeding.

After 48 hours the veliger larvae, fully protected by their shells, were collected by passing the contents of each culture jar through a 325-mesh stainless steel screen. The screen retained the shelled veligers but permitted undeveloped eggs, and embryos that had not progressed to the shelled stage, to pass through. The normal larvae from all culture jars were thus collected and pooled in 4 to 7 liters of sea water in a tall narrow jar. After thoroughly mixing the contents of this jar with a perforated plastic plunger, to obtain a uniform distribution of the larvae, a sample was withdrawn and the number of larvae in one ml. of the pooled culture determined. By keeping the pooled culture thoroughly mixed, with the larvae uniformly suspended, and taking appropriate volumes, the desired number of larvae could be introduced into each culture. The larvae of all cultures were, therefore, closely comparable in size and quality, as well as number, when the different food treatments were started on the second day.

All culture jars were kept covered so they were uniformly dark and all were in a common water bath. Although the temperature of the water bath fluctuated between 21.0° and 23.0° C. all cultures were equally affected. The sea water in all cultures was replaced with fresh cotton-filtered sea water every second day (Loosanoff and Davis, 1950).

Neither aeration nor mechanical agitation is necessary for normal development of oyster larvae when the water is changed every second day. We did not, therefore, use either aeration or mechanical agitation in these experiments as it is difficult to insure equality of such treatments in a series of cultures.

Samples were taken whenever desired by first washing the entire contents of a culture jar onto the 325-mesh screen, then re-suspending the larvae in 1000 ml. of sea water in a parallel-sided graduated cylinder. To insure uniform distribution of the larvae, the contents of the cylinder were thoroughly mixed with a perforated plastic plunger and a one-, two- or five-cc. sample was withdrawn and preserved. All the larvae in a sample were transferred to a Sedgwick-Rafter cell and examined under a compound microscope. Larvae that were living at the time the sample was taken could thus be identified and counted to determine the per cent surviving and a random sample of 100 of these larvae was measured to obtain growth data.

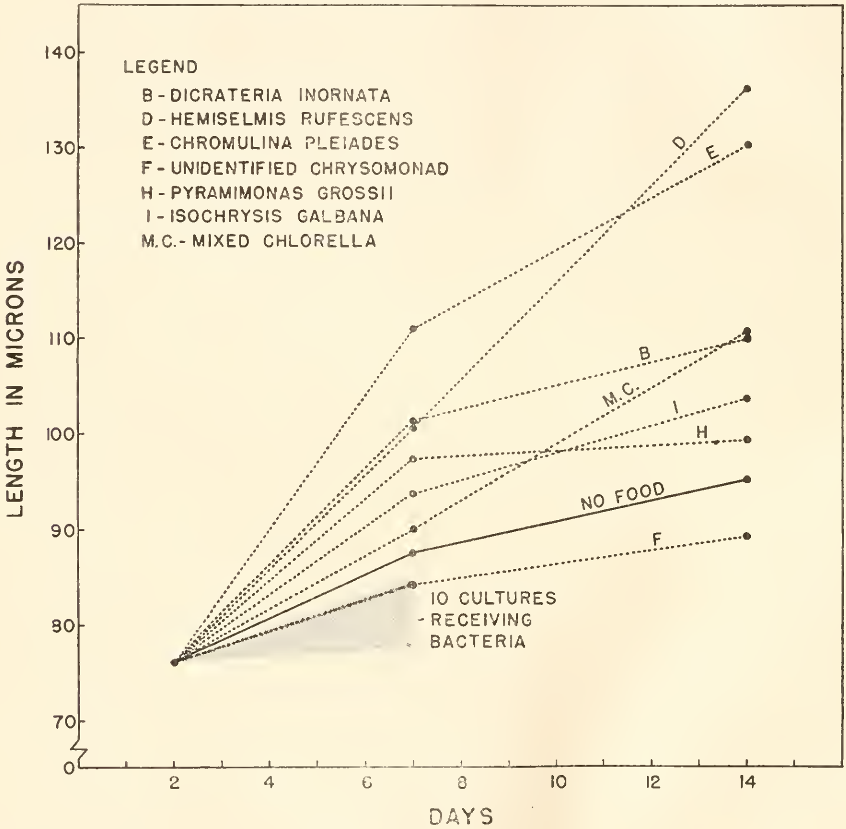


Figure 1. Growth of larvae of *C. virginica* fed different foods. Each point on a curve represents the mean length of 100 larvae. Flagellates D, E, and I were fed at the rate of 10,000 per ml./day. Flagellates H and F were fed at the rate of 5000 per ml./day. Flagellate B was fed at the rate of 10,000 per ml./day for first seven days and 5000 per ml./day thereafter.

Eighteen parallel cultures were used in the first of the present series of experiments. Each of the first nine received a different species of the marine bacteria isolated by Dr. Burkholder, six of the remaining each received a different species of the marine flagellates received from England, one received *B. coli* plus a bacteriophage, one received our "mixed Chlorella" and the final culture, which received no supplemental food, served as the control.

The larvae in all ten cultures receiving bacteria and those in the culture receiving flagellate F, an unidentified chrysomonad, grew less rapidly than did those in the unfed control culture (Fig. 1). Moreover, in the ten cultures receiving bacteria, the larvae were all dead by the eleventh day. We can assume that these bacteria and flagellate F were not utilized.

In the remaining six cultures that received supplemental food, the larvae grew more rapidly than did those in the unfed control. We can conclude, therefore, that oyster larvae probably do utilize the five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Hemiselmis rufescens, Isochrysis galbana* and *Pyramimonas grossii,* as well as our "mixed Chlorella."

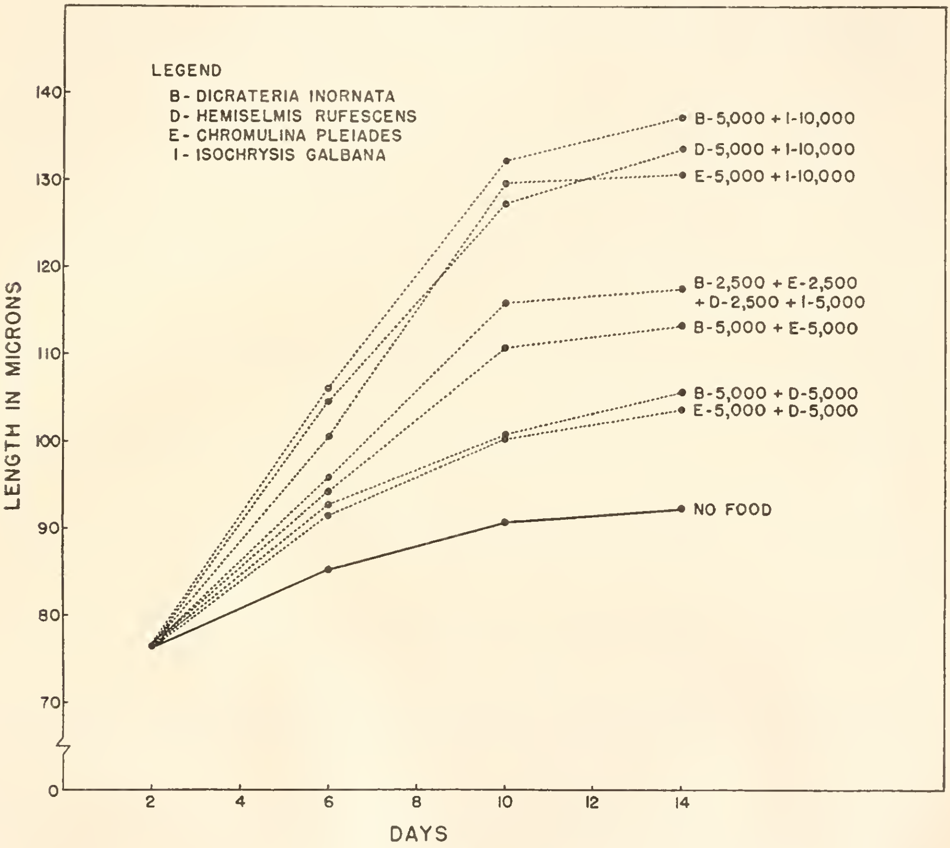


FIGURE 2. Growth of larvae of *C. virginica* fed combinations of flagellates. Each point on a curve represents the mean length of 100 larvae from each of duplicate cultures. Numbers following letter designation of flagellates indicate rate of feeding in number of flagellates per ml./day.

Unfortunately, due to difficulties in mass production of the flagellates, we were not able to feed equal numbers of each flagellate nor were we able to keep the rate of feeding constant in the larval culture receiving flagellate B (Dicrateria).

In this culture Dicrateria was added at the rate of 10,000 cells per ml./day for the first seven days but after that it was necessary to reduce the rate to 5000 cells per ml./day.

Flagellate I (Isochrysis) and flagellate D (Hemiselmis) were added at the rate of 10,000 cells per ml./day throughout the experiment, while flagellates F (the unidentified chrysomonad) and H (Pyramimonas) were added at the rate of 5000 cells per ml./day. Moreover, since this was a preliminary experiment, single cultures were used and the results are consequently less reliable than in later experiments where duplicate or triplicate cultures were used to test each food. Isochrysis, for example, appears to be a comparatively much poorer food here than it proved to be in later experiments. Note that the larvae fed "mixed Chlorella" and those fed flagellate D (Hemiselmis) grew slightly faster after the

TABLE I

*Mean length of larvae (in microns), receiving different food treatments, at 6, 10 and 14 days with growth increments calculated as mean length minus mean length of 2-day-old larvae (76.5μ)*

| Food treatment | Mean length 6 days | Growth increment 2–6 days | Mean growth increment | Mean length 10 days | Growth increment 2–10 days | Mean growth increment | Mean length 14 days | Growth increment 2–14 days | Mean growth increment |
|---|---|---|---|---|---|---|---|---|---|
| No food | | | | | | | | | |
| Culture No. 1 | 85.90 | 9.40 | | 92.40 | 15.90 | | 93.25 | 16.75 | |
| Culture No. 2 | 84.60 | 8.10 | 8.750 | 89.30 | 12.80 | 14.350 | 91.35 | 14.85 | 15.800 |
| B+E | | | | | | | | | |
| Culture No. 1 | 95.60 | 19.10 | | 112.20 | 35.70 | | 113.35 | 36.85 | |
| Culture No. 2 | 92.75 | 16.75 | 17.925 | 109.35 | 32.85 | 34.275 | 113.30 | 36.80 | 36.825 |
| B+D | | | | | | | | | |
| Culture No. 1 | 93.80 | 17.30 | | 100.60 | 24.10 | | 102.83 | 26.35 | |
| Culture No. 2 | 91.85 | 15.35 | 16.325 | 100.75 | 24.25 | 24.175 | 108.50 | 32.00 | 29.175 |
| B+I | | | | | | | | | |
| Culture No. 1 | 106.65 | 30.15 | | 133.75 | 57.25 | | 139.60 | 63.10 | |
| Culture No. 2 | 105.40 | 28.90 | 29.525 | 130.55 | 54.05 | 55.650 | 134.75 | 58.25 | 60.675 |
| E+D | | | | | | | | | |
| Culture No. 1 | 91.95 | 15.45 | | 99.25 | 22.75 | | 103.70 | 27.20 | |
| Culture No. 2 | 90.85 | 14.35 | 14.900 | 101.70 | 25.20 | 23.975 | 103.95 | 27.45 | 27.325 |
| E+I | | | | | | | | | |
| Culture No. 1 | 98.65 | 22.15 | | 127.10 | 50.60 | | 129.95 | 53.45 | |
| Culture No. 2 | 102.35 | 25.85 | 24.000 | 132.25 | 55.75 | 53.175 | 131.35 | 54.85 | 54.150 |
| D+I | | | | | | | | | |
| Culture No. 1 | 104.30 | 27.80 | | 125.00 | 48.50 | | 131.20 | 54.70 | |
| Culture No. 2 | 104.95 | 28.45 | 28.125 | 129.40 | 52.90 | 50.700 | 135.75 | 59.25 | 56.975 |
| B+E+D+I | | | | | | | | | |
| Culture No. 1 | 94.75 | 18.25 | | 116.05 | 39.55 | | 116.90 | 40.40 | |
| Culture No. 2 | 97.05 | 20.55 | 19.400 | 114.70 | 38.20 | 38.875 | 117.75 | 41.25 | 40.825 |

seventh day, while those receiving other foods grew somewhat less rapidly the second week (Fig. 1). This may indicate that these two forms are more readily utilized by the larvae in the later stages of development.

Subsequently, in experiments of factorial design, we have fed oyster larvae various combinations of those flagellates which the previous experiment had shown the larvae could utilize. We hoped to estimate the relative value of the different flagellates as foods for oyster larvae, and to determine whether some combination of them might provide a more balanced diet which would result in more rapid larval growth.

The results of a typical experiment of this kind show that certain combinations did appear to give more rapid growth of larvae than others (Fig. 2). The combination of the flagellates B + E, for example, gave more rapid growth than either the combination B + D or E + D. Nevertheless, the greatest differences are correlated with the total number of flagellates given per day. Thus, the three combinations B + I, D + I, or E + I, each consisting of a total of 15,000 flagellates per ml./day gave definitely more rapid growth than did the combination B + E + D + I which consisted of a total of only 12,500 cells per ml./day. Moreover, this latter combination in turn gave slightly more rapid growth than did any of those combinations consisting of only 10,000 flagellates per ml./day. Thus, the total number of flagellates added appears to be an important factor and suggests that the different species of flagellates may be of nearly equal value as foods for oyster larvae.

TABLE II

*Ratios of the mean growth increments, due to each food combination, to the mean growth increments of larvae in the unfed control cultures. (Standard error of averages 3.75%)*

| Days | No food | B+E | B+D | B+I | E+D | E+I | D+I | B+E+D+I |
|---|---|---|---|---|---|---|---|---|
| 2–6 | 1 | 2.049 | 1.866 | 3.374 | 1.703 | 2.743 | 3.214 | 2.217 |
| 2–10 | 1 | 2.389 | 1.685 | 3.878 | 1.671 | 3.706 | 3.533 | 2.709 |
| 2–14 | 1 | 2.331 | 1.847 | 3.840 | 1.729 | 3.427 | 3.606 | 2.584 |
| Average | 1 | 2.256 | 1.799 | 3.697 | 1.701 | 3.292 | 3.451 | 2.503 |

Statistical tests showed that the differences in rate of larval growth, attributable to differences in the total number of flagellates present, were indeed significant. Nevertheless, to account completely for the results observed it is also necessary to assume that the different species of flagellates were not of equal value as foods for oyster larvae.

An analysis of variance of the growth increments (Table I) showed that the average difference between growth increments of duplicate cultures was not significant. We are justified, therefore, in using the ratio of the mean growth increments of the duplicate cultures as a relative measure of the efficiency of the different foods. Dividing the mean growth increments for each period by the mean growth increment of the unfed cultures for the same period gives relatively constant ratios (Table II). An analysis of variance showed that differences between the ratios for the 2–6-, 2–10- and 2–14-day periods were not significant. The average ratio can be used, therefore, as a numerical estimate of the relative over-all efficiency of the different food combinations at the particular concentrations used in this experiment.

This suggests that if the effect on growth of larvae on any given food really is constant, the uncontrolled factors responsible for the growth of unfed cultures have a multiplicative power on the effect of the different food combinations. If we postulate, in addition, that the effect of each species of flagellate is proportional to its concentration and independent of the presence or absence of other foods, we can formulate the following equation for the growth increment (Y) of any combination of foods: $Y = k\ (1 + b + e + \ldots)$, where k is the uncontrolled variable responsible for the growth of unfed cultures and b, e, . . . are the effects of the foods B, E, etc. Thus, if b is the effect of 2500 *B* (Dicrateria) per cc., e is the effect of 2500 E (Chromulina) per cc., d is the effect of 2500 D (Hemiselmis) per cc. and i is the effect of 5000 I (Isochrysis) per cc., the equation for the growth increment of the food combination $B + E + D + I$ becomes $Y = k\ (1 + b + e + d + i)$, while for the combination $B + E$, in this experiment it becomes $Y = k\ (1 + 2b + 2e)$ and for the unfed cultures is $Y = k\ (1 + 0)$ or $Y = k$. From the design of the experiment and having a value for k (the observed growth increment of unfed cultures) we can calculate values for b, e, d and i using the values given for the food combinations in the average ratio (Table II). Thus b, the effect of 2500 Dicrateria per cc., becomes: $b = 1/7\{[(2.256-1) + (1.799-1) + (3.697-1) + (2.503-1)] - 3/4[(1.701-1) + (3.292-1) + (3.451-1)]\}$ or $b = 1/7(6.255-4.083) = 0.3103$.

Similarly, the separate effects of the other flagellates become $e = 0.185$, $d = 0.110$, and $i = 0.982$, but this ratio compares 2500 of each B, E and D with 5000 of I. When all are adjusted to the 10,000 level so that we compare the flagellates cell for cell, they become $b = 1.240$, $e = 0.740$, $d = 0.440$ and $i = 1.964$. Since these values were taken from the average ratio of the food combinations, the values represent the over-all efficiency of the foods throughout the experiment.

Similar ratios or estimates of food values can be worked out for the separate four-day periods; corrected by successive approximations and adjusted to the 10,000 level for each flagellate they are:

| | b | e | d | i |
|---|---|---|---|---|
| 2–6 days | 1.372 | 0.788 | 0.776 | 1.716 |
| 6–10 days | 1.616 | 1.112 | −0.820 | 3.226 |
| 10–14 days | 3.776 | −2.504 | 5.123 | 0.450 |

The value of most of the flagellates varies considerably. These variations probably represent day-to-day fluctuations in both physiological state and purity of the flagellate cultures used as foods, for it is known that in many micro-organisms the chemical composition varies with their physiological condition. Thus, although B (Dicrateria), in this experiment, appears to be a better food than E (Chromulina) in approximately 50 per cent of the experiments of this series the reverse is true. It seems probable, therefore, that differences in rate of growth of oyster larvae brought about by variations in the physiological condition of Chromulina and Dicrateria are as great or greater than differences dependent upon which of these two species the oyster larvae are fed.

Flagellate D (Hemiselmis) appears to be the poorest of these four species. However, the high value for the 10–14-day period may indicate, as in the previous experiment, that Hemiselmis is more readily utilized by older larvae. Unfortunately, this flagellate culture was lost and we could not obtain further data on its effect on the rate of growth of oyster larvae.

Bruce, Knight and Parke (1940), in feeding experiments on larvae of the European oyster, *Ostrea edulis,* rated flagellate I as a "good to very good" food while rating flagellate B as "fair." Although their tabulated data seem to indicate that flagellate F was utilized and that flagellate D was not, the authors do not discuss these two flagellates further.

The second part of the problem was to determine whether, by combining the various flagellates, we could provide a diet that would bring about more rapid growth of oyster larvae than could be obtained by feeding an equivalent number of cells of a single species. From a statistical viewpoint, if the separate growth increments due to the different foods, as calculated above, can be combined according to the formula to give a satisfactory fit to the growth increment observed when these foods are used in combination, we will have no reason to believe that there are interactions. In other words, if the effect of B and the effect of E can be added together as in the formula to give a growth increment that agrees closely with that observed when B and E were fed in combination, we will have no reason to believe that the combination B + E is any better as a food than equivalent quantities of B or E alone.

TABLE III

*Differences between observed and calculated mean lengths of oyster larvae receiving different foods*

| Foods | 6 days | | | 10 days | | | 14 days | | |
|---|---|---|---|---|---|---|---|---|---|
| | Observed | Calculated | Diff. | Observed | Calculated | Diff. | Observed | Calculated | Diff. |
| No food | 85.2 | 84.8 | −0.4 | 90.9 | 90.9 | 0.0 | 92.3 | 92.3 | 0.0 |
| B+E | 94.2 | 93.7 | −0.5 | 110.2 | 110.3 | +0.1 | 113.3 | 113.2 | −0.1 |
| B+D | 92.8 | 93.7 | +0.9 | 100.7 | 100.7 | 0.0 | 105.7 | 108.8 | +3.1 |
| B+I | 106.0 | 104.7 | −1.3 | 132.2 | 134.3 | +2.1 | 137.2 | 137.1 | −0.1 |
| E+D | 91.4 | 91.3 | −0.1 | 100.5 | 100.6 | +0.1 | 103.8 | 103.9 | +0.1 |
| E+I | 100.5 | 102.3 | +1.8 | 129.7 | 130.2 | +0.5 | 130.7 | 130.0 | −0.7 |
| D+I | 104.6 | 102.2 | −2.4 | 127.2 | 126.1 | −1.1 | 133.5 | 133.2 | −0.3 |
| B+E+D+I | 95.9 | 98.0 | +2.1 | 115.4 | 114.7 | −0.7 | 117.3 | 119.6 | +2.3 |

A statistical analysis of the results of the previous experiment shows no evidence that any of the combinations of flagellates tested results in more rapid growth of oyster larvae than would equivalent amounts of any of the flagellates separately. The observed mean lengths of larvae, receiving combinations of the different flagellates as foods, were compared with the mean length calculated from the values of the separate foods given above as the sum of the growth increment given by the formula plus the mean length of larvae in the unfed control cultures (Table III). At six days the maximum difference between observed and calculated mean length is 2.4 $\mu$, at 10 days it is 2.1 $\mu$, and at 14 days it is 3.1 $\mu$. These differences are of the same order of magnitude as differences between parallel cultures receiving the same treatment and it can be shown that the fit between calculated and observed mean lengths is satisfactory. Therefore, we have no reason to believe that the foods are not completely additive, and no interaction is indicated. Bruce, Knight and Parke (1940) believed that a combination of the flagellates H and I was especially suitable as food for larvae of *O. edulis* but did not test for an interaction. Thus far we have not tested this combination with larvae of *C. virginica.*

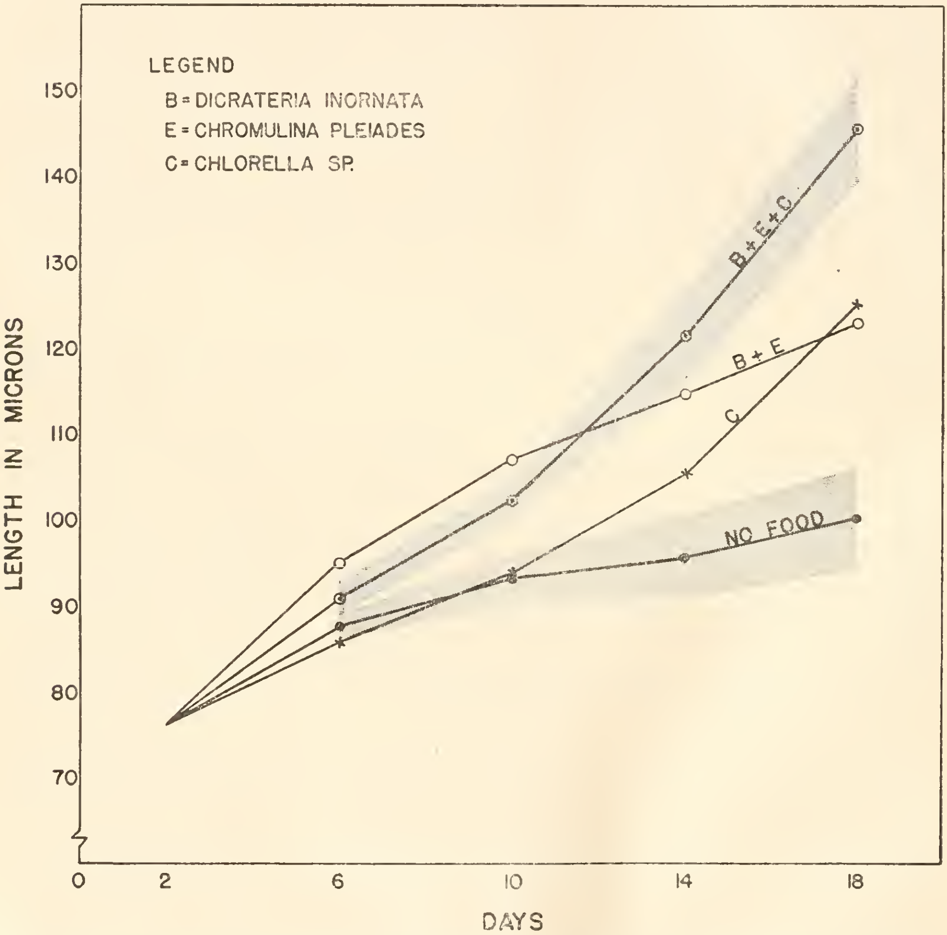


FIGURE 3. Growth of larvae of *C. virginica* fed *Chlorella* sp. alone and in combination with flagellates. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures. Shaded areas represent 95 per cent confidence bands. Flagellates B and E fed at the rate of 5000 per ml./day and *Chlorella* sp. fed at the rate of 50,000 per ml./day.

Studies to determine what concentration of any single species of these flagellates would be required to induce the maximum rate of growth of oyster larvae are still in progress. Preliminary tests indicate, however, with Dicrateria alone as the supplementary food, that this flagellate must be fed at the rate of 25,000 cells per ml./day (the highest rate tested), or higher, to produce the most rapid growth of larvae possible in cultures such as ours, which contain approximately 5000 larvae per liter. Isochrysis has not been tested in varying concentrations, but the larvae grew rapidly when this flagellate was fed at the rate of 20,000 cells per ml./day. When Chromulina was used as the supplemental food, however, the larvae grew slightly faster in cultures receiving 15,000 cells per ml./day than in cultures receiving 20,000 cells per ml./day.

Our experiments indicate, therefore, that while the concentration of flagellates required to give the most rapid growth of larvae probably varies with the species of flagellate, none of the flagellates that were utilizable gave toxic effects when added to our larval cultures at rates up to 15,000 cells per ml./day and for certain species up to 25,000 cells per ml./day. Korringa (1949), on the other hand, although he obtained good growth of larvae and a heavy spatfall of *O. edulis* in his 1947 experiments with concentrations of flagellates between 10,000 and 20,000 per ml., concluded from later experiments that (p. 4) "water initially containing more than 5000 flagellates, or a commensurable great number of other phytoplankton, should be mistrusted as it may contain toxic concentrations of phytoplankton metabolites from the first day the tanks are filled." Imai and Hatanaka (1949) indicate that in culturing larvae of *Crassostrea gigas,* they strive to keep the concentration of the flagellate, *Monas* sp., at a concentration of only 1000 to 2000 per ml. in their cultures. These authors have, at maximum, only about 200 larvae per liter, however, and such low concentrations of flagellates would provide too small a quantity of food to induce appreciable growth of larvae in cultures such as ours with a concentration of approximately 5000 larvae per liter.

In other experiments we sought to determine the effect of the bacteria-free culture of *Chlorella* sp. on the rate of growth of oyster larvae. In these experiments *Chlorella* sp. was tested both alone and in combination with some of the flagellates (Fig. 3). Using triplicate cultures for each treatment, one trio served as a control and received no supplementary food, one trio received *Chlorella* sp. alone (50,000 per ml./day), one trio received a combination of flagellates B + E (5000 per ml./day of each) and one trio received *Chlorella* sp. (50,000 per ml./day) in addition to the combination of flagellates B + E (5000 per ml./day of each).

Our "mixed Chlorella" culture, although apparently being more effectively used by larvae in the later stages of development, had been utilized from the start (Fig. 1). With a pure culture of Chlorella, however, in this and in several repetitions of the experiment, the effect on the growth rate of the larvae during the early stages, although small, is consistently negative (Fig. 3). This is true both when pure Chlorella is added alone, and when it is used in combination with the flagellates. Some time between the sixth and twelfth days, however, the larvae appear to become able to utilize *Chlorella* sp. and it accelerates their growth markedly.

Calculations similar to those previously mentioned give the following values for b, e and c (effect of *Chlorella* sp.):

| | b | e | c |
|---|---|---|---|
| 2–6 days | 0.252 | 0.329 | −0.164 |
| 6–10 days | 0.191 | 0.579 | 0.323 |
| 10–14 days | 0.598 | 0.580 | 2.364 |
| 14–18 days | 0.620 | 0.630 | 3.467 |

However, when these figures are adjusted to the 10,000 level so that we are comparing food micro-organisms cell for cell, it becomes obvious that Chlorella is inferior to the flagellates as a food on such a basis, thus:

| | b | e | c |
|---|---|---|---|
| 2–6 days | 0.504 | 0.658 | −0.033 |
| 6–10 days | 0.382 | 1.158 | 0.065 |
| 10–14 days | 1.196 | 1.160 | 0.473 |
| 14–18 days | 1.240 | 1.260 | 0.693 |

Again there is no evidence of an interaction of food organisms and the growth increment of the combination B + E + Chlorella is given by $Y = k(1 + b + e + c)$. These experiments explain our previously published observations (Davis, 1950a) that "mixed Chlorella," while not consistently a good food during earlier larval stages, served quite well as food during later larval stages. The good growth obtained during later larval stages is undoubtedly due to the utilization of Chlorella itself by the oyster larvae. The inconsistency of growth of earlier larval stages is understandable since, from these experiments, we know that during these stages the larvae do not utilize Chlorella. Obviously, then, growth during the early larval stages, when "mixed Chlorella" was used as a food, was due to other forms which might or might not be present at any given time in our mass culture of Chlorella, and to small amounts of food in the sea water, which may, as in the experiment shown in Figure 3, carry the larvae through the early stages until they can utilize Chlorella.

Cole (1936) after a review of the literature on the European oyster (*O. edulis*) states that the conclusion that the larvae are able to develop on Chlorella is not supported by results of well designed critical experiments. Presumably the larvae are unable to digest this alga. We have shown here, however, that Chlorella is utilized by older larvae of *C. virginica* and we have unpublished data showing that larvae of *Ostrea lurida*, a species closely related to *O. edulis*, can be reared to metamorphosis on either mixed Chlorella or a bacteria-free culture of Chlorella.

A number of cultures of *C. gigas* have been reared to metamorphosis (Davis, 1950b) using our "mixed Chlorella" as a food, although in general larvae of this species are similar to larvae of *C. virginica* in being limited as to the types of micro-organisms they can utilize as foods. In addition Loosanoff and Davis (1950), Loosanoff, Miller and Smith (1951), and Loosanoff and Marak (1951) have reared *O. edulis, Venus mercenaria, Mya arenaria* and several other species of lamellibranch larvae using our "mixed Chlorella" as the chief food. Such results certainly indicate that the ability to utilize Chlorella as a food is common to several species of lamellibranch larvae.

In the course of our experiments certain differences in rate of growth of oyster larvae, in different members of a trio or pair of cultures receiving the same treatment, appeared to be due to differences in the number of larvae present in the culture. An experiment in which triplicate cultures at each of four different con-

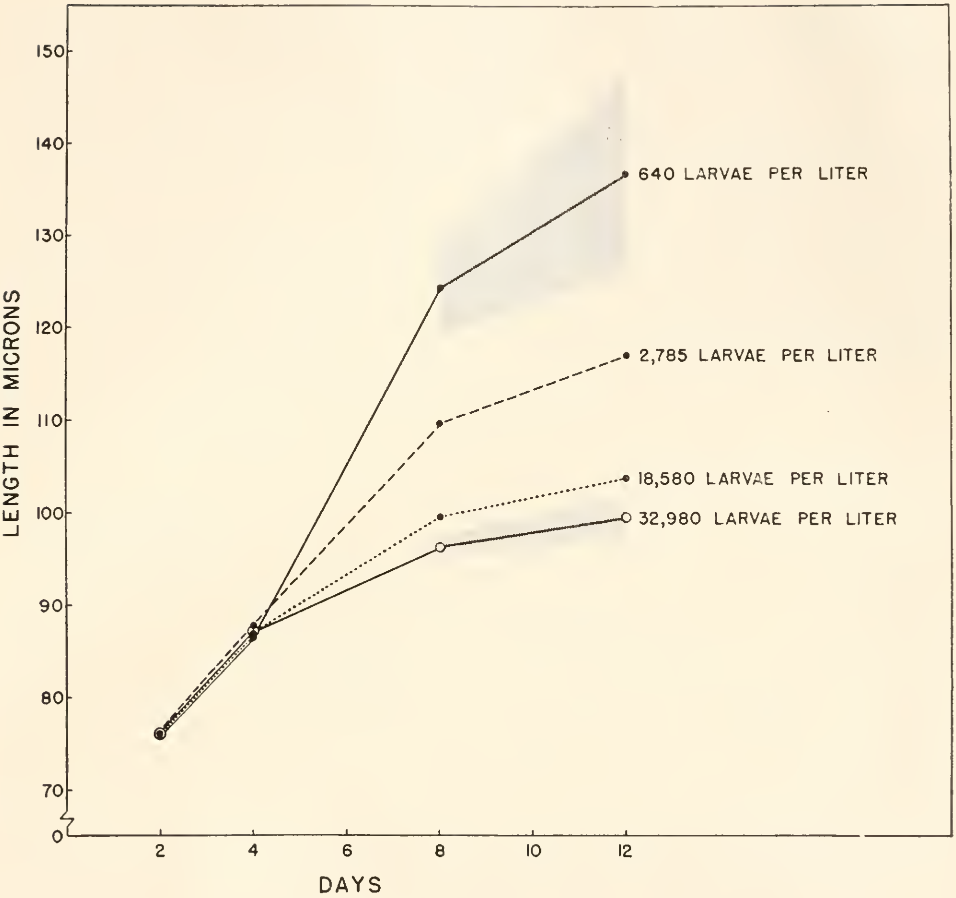


FIGURE 4. Growth of larvae of *C. virginica* cultured at four different concentrations with food constant at 50,000 cells per ml./day. Shaded areas represent 95 per cent confidence bands. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures.

centrations of larvae were used indicated an inverse relation between the concentration of larvae and their rates of growth (Fig. 4). These cultures all received 50,000 cells per ml./day of our "mixed Chlorella" as a supplementary food.

The between-culture variations, at the two lower concentrations of larvae, were abnormally great, while at the higher concentrations the between-culture variations were normal. Thus at 14 days the average sizes of larvae in the various cultures were as follows:

| Number of larvae per liter | Culture number 1 | Culture number 2 | Culture number 3 |
|---|---|---|---|
| 640 | 140.0 | 140.1 | 125.17 |
| 2,785 | 110.50 | 111.57 | 126.55 |
| 18,580 | 102.65 | 103.35 | 105.35 |
| 32,980 | 98.35 | 98.70 | 101.60 |

The 95 per cent confidence bands (Fig. 4) were calculated with the inconsistent values included. We are probably justified in concluding that with all cultures receiving equal quantities of this food, there is an inverse relation between the concentration of larvae in a culture and their rate of growth, at least after the eighth day. The failure of the inverse relation to appear earlier is probably due to the inability of oyster larvae to utilize the "mixed Chlorella" readily during the earlier stages of development.

We do not know whether similar results would be obtained with other foods, but suspect that they would be, nor do we know what results would be obtained if the quantity of food were kept proportional to the concentration of larvae. The results of the experiment suggest, however, that the concentration of larvae in a culture must be considered in comparing rates of larval growth.

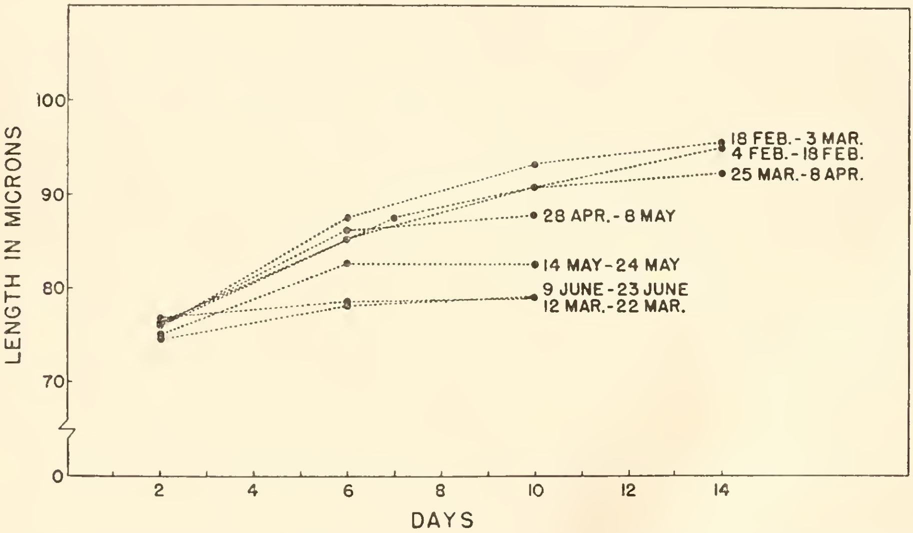


FIGURE 5. Growth curves of unfed control cultures of *C. virginica* larvae showing variations between seven successive experiments. Each point on a curve represents the mean length of 100 larvae from each of duplicate or triplicate cultures.

Other differences, which make comparison of rates of larval growth difficult, are those between successive cultures in which the larvae receive identical treatments. The factor or factors (k) responsible for these differences affect not only the rate of growth of larvae but also, in some experiments, their survival as well. The growth curves of the larvae in the unfed control cultures of seven successive experiments illustrate these differences (Fig. 5). In the 1952 experiments of February 18–March 8 and of February 4–18, the curves appear normal since growth of the larvae in the unfed cultures, although slow, was continuous throughout the 14 days of the experiments. The growth of larvae appears subnormal throughout the May 14–24 experiment, when compared to the above curves, and in the experiments of March 12–22 and June 9–19 almost no growth occurred in these unfed cultures. In the experiment of March 25–April 8 the flattening of the curve between the tenth and fourteenth days, which reflects an almost complete lack of growth of the larvae during this period, appears abnormal. In the experiments of April 28–May 8 and May 14–24, similar abnormal flattening of the growth curves occurred between the sixth and tenth days.

In each of the three experiments in which the flattening of the growth curves of the unfed cultures occurred after the sixth day, there were also seven pairs of cultures receiving different foods, yet all cultures showed similar flattening of their growth curves simultaneously.

This flattening of growth curves cannot be considered a normal deceleration of growth since it is not correlated with either age of larvae (Fig. 5), or with size (Figs. 2 and 5). Moreover, a pronounced simultaneous upswing of growth curves in all cultures was noted in one experiment and minor simultaneous upward trends were noted in several others. Such simultaneous changes in the growth curves of the larvae in all cultures of an experiment, regardless of food treatment, must be a reflection of variation in some unknown factor or factors common to all cultures. The factors common to all cultures, and least susceptible to control, are the physical, chemical and microbiological constituents of the sea water in which the larvae were reared.

Also strongly suggestive of a variation in the physical or chemical constituents of the sea water was the variation in peak densities of flagellate cultures grown in media prepared from sterilized sea water enriched with constant amounts of nutrient salts. Culture media prepared from sea water taken during periods of poor larval growth gave lower peak densities of flagellates than did media made up from sea water taken during periods of good larval growth.

In all of our experiments, in cultures that received flagellates known to be utilizable, the larvae have grown more rapidly than those in the parallel unfed control cultures. Yet we have not been able to overcome completely the effects of the unknown factor (or factors) by supplemental feeding. For example, under the conditions existing during the May 14–24 and June 9–19 experiments, feeding a combination of the flagellates Isochrysis and Dicrateria produced only about 1/5 as much growth of larvae, in ten days, as did identical concentrations of the same combination of flagellates in the experiment of March 25–April 8. This suggests that one phase of the action of the unknown factor may be to affect the ability of the larvae to utilize the food that is available.

Several authors have suspected unidentified variations in sea water of affecting their results. Loosanoff, Miller and Smith (1951) noted a lack of uniformity of results in consecutive experiments with larvae of *Venus mercenaria* and considered it possible (p. 75) "that at different times the water itself contained certain dissolved substances which, in a manner not yet understood, affected the rate of development of bivalve larvae." Wilson (1951), working in England with polychaete larvae, found a difference between sea water from two different areas and attributed the poor growth of larvae in water from one of these sources to a lack of (p. 18) "some unknown constituent, essential for healthy development" of the species of polychaetes he used. One of these unidentified variants may be that described by Collier, Ray and Magnitzky (1950) who reported a substance in sea water that can be measured photometrically with N-ethyl carbazole, the concentration of which could be correlated with the pumping rate of oysters.

Wangersky (1952) reported that the substance, measured photometrically with N-ethyl carbazole, was a mixture of dehydroascorbic acid and a rhamnoside. He concludes (p. 685) "that the vitamin is present in the sea largely in the form of dehydroascorbic acid." Although we have not yet tried dehydroascorbic acid, we have added ascorbic acid to the sea water in which the larvae were reared. This did not improve the rate of growth of oyster larvae, but merely resulted in a dense growth of bacteria which killed the larvae. Similar exploratory experiments indicate that additions of Vitamin $B_{12}$, iodine, $Mn^{++}$, $Fe^{+++}$, $Zn^{++}$ or $PO_4^{---}$, likewise, do not improve the rate of growth of oyster larvae.

To verify that deleterious changes in the sea water in our laboratory system were not causing the poor growth and high mortalities of our June cultures, an experiment was designed to compare the laboratory sea water with sea water taken directly from Milford Harbor in enamel buckets. Parallel cultures of oyster larvae were started, two in sea water from each source. In addition a single culture of larvae of *Venus mercenaria* was started in laboratory sea water at the same time. All cultures received approximately 50,000 cells per ml./day of the "mixed Chlorella" as food.

At 14 days all the oyster larvae in sea water from both sources were dead, while the Venus larvae still appeared healthy and were growing normally. At 18 days the Venus larvae had reached setting size and a count revealed that approximately 55 per cent of the larvae that had been counted 12 days earlier while still in the straight hinge stage were still living. Thus, although growth was somewhat slower than average (Loosanoff and Davis, 1950; Loosanoff, Miller and Smith, 1951) the Venus larvae had lived and grown to setting stage, as in several previous experiments (Loosanoff, 1950), under conditions in which the oyster larvae had all died.

In other experiments water from points several miles offshore in Long Island Sound was used but again no significant difference in rate of growth of oyster larvae was noted. This experiment indicated that the "water factor" that resulted in poor larval growth was not just a local condition. We agree, therefore, with Wilson (1951), who concluded (p. 18) "it is evident that many animals find no difficulty in living and reproducing under water conditions that seem to affect some other species adversely."

We do not yet know what this "water condition" is, nor whether it is the presence of some inhibitory or toxic substance that causes poor growth, or, as Wilson (1951) believes, it is the lack of something necessary for good growth. Our experiments do indicate that one phase of its action is to affect the ability of larvae of *C. virginica* to utilize the food that is present.

The author wishes to express his gratitude to Dr. F. S. Russell of the Plymouth Laboratory of the Marine Biological Association of U. K. for sending us innocula of the six species of marine flagellates cultured by Dr. Mary Parke; to Dr. P. R. Burkholder of Yale University who isolated the nine species of marine bacteria used; and to Dr. Ralph Lewin of Yale University who isolated the *Chlorella* sp. from our mixed phytoplankton culture. The author is especially indebted to Dr. V. L. Loosanoff not only for obtaining the pure cultures of micro-organisms but also for constructive criticism throughout the experimental work and the preparation of this paper. Thanks are also due my co-workers, D. W. Calhoun, who gave valuable assistance in the statistical analysis of the data, and to W. S. Miller and C. A. Nomejko for assistance in other phases of the work.

## SUMMARY

1. None of the 13 species of marine bacteria tested to date was utilized as food by oyster larvae.

2. Five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Isochrysis galbana, Hemiselmis rufescens* and *Pyramimonas grossii,* were utilized as food by oyster larvae, while another, an unclassified chrysomonad, in addition to the three flagellates previously reported, was not.

3. *Chlorella* sp. was not utilized as food by young oyster larvae but was utilized during later larval stages.

4. None of the combinations of foods tried gave any evidence of providing a more balanced diet or more rapid larval growth than could be obtained by feeding equivalent quantities of a single food. The effects of all foods tested, including Chlorella, are additive.

5. When equal numbers of cells are fed, different species of flagellates induce different rates of growth of oyster larvae.

6. Species of flagellates also differ in the number of cells needed to induce the maximum rate of growth of oyster larvae.

7. The maximum concentration of food organisms that can be created in water containing oyster larvae without unfavorably affecting the larvae varies with the species.

8. With the number of food organisms equal, the rate of growth of oyster larvae had an inverse relation to the number of larvae per unit volume.

9. Variations between rates of growth of larvae in cultures receiving the same treatment in successive experiments appear to be due to some variable factor in the sea water that affects the ability of the larvae to utilize the food that is present.

## LITERATURE CITED

BRUCE, J. R., MARGERY KNIGHT AND MARY W. PARKE, 1940. The rearing of oyster larvae on an algal diet. *J. Mar. Biol. Assoc. U. K.,* **24**: 337–373.

COLE, H. A., 1936. Experiments in the breeding of oysters (*Ostrea edulis*) in tanks, with special reference to the food of the larvae and spat. *Min. Agri. & Fish., Fisheries Investigations Series II,* **15**: 1–28.

COLLIER, A., S. RAY AND W. MAGNITZKY, 1950. A preliminary note on naturally occurring organic substances in sea water affecting the feeding of oysters. *Science,* **111**: 151–152.

DAVIS, H. C., 1950a. On food requirements of larvae of *Ostrea virginica.* *Anat. Rec.,* **108**: 132–133.

DAVIS, H. C., 1950b. On interspecific hybridization in Ostrea. *Science,* **111**: 522.

IMAI, T., AND M. HATANAKA, 1949. On the artificial propagation of Japanese common oyster, *Ostrea gigas,* Thun. by non-colored naked flagellates. *Bull. Inst. of Agric. Res., Tohoku University,* **1**: 33–46.

KORRINGA, P., 1949. Difficulties encountered in tank breeding of oysters. *Special Scientific Meeting on Shellfish. International Council for the Exploration of the Sea, October 1949.*

Loosanoff, V. L., 1945. Precocious gonad development in oysters induced in midwinter by high temperature. *Science,* **102**: 124–125.

Loosanoff, V. L., 1950. Variations in Long Island Sound oyster set. *Atlantic Fisherman,* **30**: 15 and 47.

Loosanoff, V. L., and H. C. Davis, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.,* **98**: 60–65.

Loosanoff, V. L., and H. C. Davis, 1952. Temperature requirements for maturation of gonads of northern oysters. *Biol. Bull.,* **103**: 80–96.

Loosanoff, V. L., and R. R. Marak, 1951. Culturing lamellibranch larvae. *Anat. Rec.,* **111**: 129–130.

Loosanoff, V. L., W. S. Miller and P. B. Smith, 1951. Growth and setting of larvae of *Venus mercenaria* in relation to temperature. *J. Mar. Res.,* **10**: 59–81.

Wangersky, Peter J., 1952. Isolation of ascorbic acid and rhamnosides from sea water. *Science,* **115**: 685.

Wilson, D. P., 1951. A biological difference between natural sea waters. *J. Mar. Biol. Assoc. U. K.,* **30**: 1–19.

# THE EFFECT OF X-IRRADIATION ON NUCLEASE ACTIVITY AND RESPIRATION OF TETRAHYMENA GELEII W[1]

HERBERT J. EICHEL AND JAY S. ROTH

*William Goldman Isotope Laboratory, Division of Biological Chemistry, The Hahnemann Medical College and Hospital, Philadelphia, Pa., and the Marine Biological Laboratory, Woods Hole, Mass.*

The marked resistance of unicellular organisms to the action of ionizing radiations, as opposed to the low resistance of mammals, has been known for some time. However, little information is available concerning the response of specific biochemical processes of single cell animals to irradiation. The process which has probably been most extensively investigated is cell respiration. Barron, Gasvoda and Flood (1949a) summarized the early papers in this field and showed that the respiration of dilute suspensions of sea urchin sperm could be inhibited from 10–66 per cent with from 100–20,000 r. The inhibition was believed to be due to the action of stable organic peroxides produced by x-irradiation of sea water (Barron *et al.*, 1949b). They also found that the utilization of succinate and acetate was impaired in x-irradiated sperm. Billen, Stapleton and Hollaender (1952) observed that a dose of 60,000 r, while decreasing the number of viable cells of a suspension of *E. coli* B/r by more than 99 per cent, had no effect on the initial respiratory rate but decreased the oxygen consumption afterwards. The presence of pyruvate or succinate was more effective than glucose in prolonging the initial period of normal activity.

As part of a general program involving the effects of x-irradiation on enzyme systems of various animals, and specifically, in an effort to extend the knowledge of biochemical changes in irradiated unicellular organisms, the enzymes ribonuclease (RNase) and desoxyribonuclease (DNase) and respiration were selected for the present study. Data obtained by Roth, Eichel *et al.* (1953) suggested that the RNase of rat liver was a highly labile enzyme under conditions of whole body irradiation.

## MATERIALS AND METHODS

*Cultures.* Pure stock cultures of *Tetrahymena geleii* W were maintained at room temperature in 50-ml. Erlenmeyer flasks containing 10–15 ml. of 2 per cent Difco proteose-peptone plus 0.2 per cent Difco yeast extract. Transfers were made each day from a 48-hour culture using a 2 mm. platinum loop. Cultures for experimental use were prepared daily by inoculating one ml. of the 48-hour stock cultures into a two-liter Erlenmeyer flask containing 750 ml. of medium.

*Preparation of cells, homogenates and dry weight determinations.* For use, the Tetrahymena from 750 ml. of 48-hour cultures were separated according to the method of Seaman (1949) using 50-ml. conical centrifuge tubes. Our cells,

---

[1] This project was supported by a grant from the Atomic Energy Commission, Contract No. (AT-30)-1069.

however, were concentrated in a volume of 25 ml. Homogenates were prepared by diluting an aliquot of the concentrate (usually 5 ml.) with an equal volume of distilled water and then grinding with silica in an all-glass homogenizer at 0–2° C. until microscopic examination revealed no unruptured cells. The homogenates were centrifuged at 5000 r.p.m. in an International No. 1 centrifuge for five minutes to remove the silica and cell fragments. Dry weight determinations were made in triplicate at 80° C. (Seaman, 1951) on the whole cells or homogenates. The dry weights of one ml. of the whole cell concentrates and one ml. of the 1:2 homogenates averaged 9.16 (6.57–13.15) and 3.68 mg. (3.00–4.04), respectively.

*Irradiation procedure.* Four ml. of the concentrated suspensions or homogenates were added to plastic containers (7/8 × 7/8 × 11/16 inches) which were then packed in ice in plastic Petri dishes to be irradiated or used as controls. The radiation factors have been described (Wichterman, 1948). The radiation flux was 6300 r per minute. When samples were irradiated at 300,000 or 500,000 r, the ice was renewed every 25 minutes.

*Respiration studies.* Oxygen consumption by the whole cells was measured at 27° C. using standard Warburg manometry. Each vessel contained one ml. of 0.1 *M* $Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7.4, one ml. of cell concentrate and 0.8 ml. of distilled water in the main compartment and 0.2 ml. of 10 per cent NaOH in the center well. The vessels were allowed to equilibrate 10 minutes, the stopcocks closed and readings taken at either 10- or 15-minute intervals for one hour. The vessels were shaken at a rate of 120 oscillations per minute and the gas phase was air. Generally, 20–30 minutes elapsed between the completion of irradiation and the first reading. The average $QO_2$ value of normal cells obtained by assay of 18 different cultures was 25.9 (16.5–34.1), while the average oxygen consumption per million cells was 109 $mm.^3$ (80–150).

*Nuclease determinations.* RNase and DNase in homogenates of Tetrahymena were assayed at 25° C. by a modification of the turbidimetric method of McCarty (1948). For the RNase tests, the system consisted of 10 ml. of 0.2 per cent sodium ribonucleate (Nutritional Biochemicals), 10 ml. of veronal-acetate buffer, pH 5.85, and a suitable volume of 1:2 homogenate (usually 2 ml.) added at zero time. In every case, the final volume was made up to 25 ml. with distilled water. The components of the DNase system were 10 ml. of 0.2 per cent sodium desoxyribonucleate containing $MgSO_4$ to give a final $Mg^{++}$ ion concentration of 0.003 *M* in 25 ml., 10 ml. of veronal-acetate buffer, pH 5.20, and 5 ml. of a 1 : 2 hemogenate. Four-ml. samples of each were withdrawn at various intervals (usually 2, 10, 20, 30, 40 and 60 minutes), added to 4 ml. of 1 *M* HCl in 18 × 150 mm. test tubes and the turbidity read after one minute in a Lumetron photoelectric colorimeter (Model 401) at 420 $m\mu$ against a distilled water blank. Under the conditions of the test, the RNase reaction rate was generally linear for 40–60 minutes while the DNase exhibited linearity during the entire 60 minutes. For each enzyme the pH employed is based on preliminary observations only, and does not unequivocally represent the pH of optimum activity. One unit of enzyme activity is defined as that amount of enzyme which, in 25 ml. of test solution, causes a decrease in optical density of 0.1 in 60 minutes. The specific activity is equal to the number of units per mg. dry weight.

## EXPERIMENTAL RESULTS

*Respiration studies.* The $QO_2$ values of normal and irradiated cells exposed to 300,000 and 500,000 r are summarized in Table I. It can be seen that respiration was usually significantly inhibited at 300,000 r, the average of twelve runs being approximately 30 per cent. No explanation can be given for the wide range of inhibition observed. In two cases, the inhibition was less than 10 per cent. In two instances assays were performed on irradiated cells after 24 hours. In one experiment, a 25 per cent increase in respiration of the irradiated cells was observed, and in the second, the increase amounted to 125 per cent. When the radiation level was raised to 500,000 r, the average inhibition was 40 per cent.

TABLE I

*The effect of x-irradiation on the respiration of Tetrahymena at 27° C.*

| Dose (r) | Time after irradiation (hrs.) | Control $QO_2$ | Irradiated $QO_2$ | Change (per cent) |
|---|---|---|---|---|
| 300,000 | 0 | 22.2 | 20.3 | − 9 |
| .... | 0 | 23.7 | 21.9 | − 8 |
| .... | 0 | 16.5 | 13.6 | −18 |
| .... | 0 | 34.0 | 25.5 | −25 |
| .... | 0 | 32.9 | 27.9 | −15 |
| .... | 0 | 23.8 | 16.7 | −30 |
| .... | 0 | 34.1* | 22.7* | −33 |
| .... | 0 | 27.8 | 17.6 | −36 |
| .... | 0 | 24.3 | 13.1 | −46 |
| .... | 0 | 30.1 | 15.7 | −48 |
| .... | 0 | 30.0 | 22.4 | −25 |
|  | 24 | 10.0 | 14.2 | +25 |
| .... | 0 | 24.4 | 12.8 | −48 |
|  | 24 | 8.0 | 18.0 | +125 |
| 500,000 | 0 | 23.3 | 12.4 | −47 |
|  | 2.5 | 25.4 | 6.2 | −76 |
| .... | 0 |  |  | −29** |
| .... | 0 | 28.8 | 16.0 | −44 |
|  | 8 | 10.8 | 2.6 | −76 |

* Data obtained with *T. geleii* strain E.

** Based on mm.$^3$ oxygen consumed.

In two instances when assays were run at 2.5 and 8 hours after exposure, the inhibition rose to 76 per cent.

It is well known that several substances will diminish radiosensitivity by interaction with active radicals produced by ionizing radiations (Patt *et al.*, 1950; Smith *et al.*, 1950; Chapman and Cronkite, 1950; Hollaender, Stapleton and Martin, 1951; Forssberg, 1950). Some of these compounds were tested for their ability to protect Tetrahymena exposed to 300,000 r. For each experiment, 2 ml. of the cell concentrates were diluted with 2 ml. of each of the neutralized test compounds, or with 2 ml. of distilled water, and then immediately irradiated. Control organisms were treated identically. After irradiating, one ml. was removed from each plastic container, added to Warburg flasks as previously described and

TABLE II

*The effect of various amino acids, glutathione and pyruvate on the respiration of control Tetrahymena and Tetrahymena exposed to 300,000 r. See text for concentration of supplement*

| Experiment No. | Supplement | Control $QO_2$ | Irradiated $QO_2$ | Inhibition (per cent) |
|---|---|---|---|---|
| 1 | —— | 27.8 | 17.6 | 36 |
| | L-cysteine | 34.4 | 28.3 | 18 |
| | L-methionine | 29.4 | 19.4 | 34 |
| 2 | —— | 30.0 | 22.4 | 25 |
| | L-cysteine | 37.0 | 30.0 | 19 |
| | Glycine | 28.9 | | |
| 3 | —— | 24.4 | 12.8 | 48 |
| | L-cysteine | 35.4 | 28.8 | 19 |
| | DL-homocysteine | 24.6 | 20.8 | 15 |
| 4 | —— | 24.3 | 13.1 | 46 |
| | L-cysteine | 34.6 | 31.8 | 8 |
| 5 | —— | 16.5 | 13.6 | 18 |
| | Glutathione | 19.0 | | |
| | Glycine | 16.5 | | |
| 6 | —— | 23.8 | 16.7 | 30 |
| | DL-serine | 26.5 | 20.1 | 24 |
| | Sodium pyruvate | 31.0 | 28.6 | 8 |

the oxygen consumption followed for one hour. The results of these studies are listed in Table II. L-cysteine ($8.3 \times 10^{-3}$ *M*) afforded 54 per cent protection (average of four experiments). In experiment 3 it can be seen that DL-homocysteine was equally effective at the same concentration. DL-serine ($16.6 \times 10^{-3}$ *M*) was only slightly effective while L-methionine ($6.7 \times 10^{-3}$ *M*) gave no pro-

TABLE III

*RNase and DNase activity of homogenates prepared from normal and irradiated cells. Figures give specific activity*

| Dose (r) | RNase | | Change (per cent) | DNase | | Change (per cent) |
|---|---|---|---|---|---|---|
| | Control | Irradiated | | Control | Irradiated | |
| 300,000 | 0.162 | 0.147 | −10 | 0.018 | 0.016 | −11 |
| .... | 0.204 | 0.204 | 0 | 0.018 | 0.018 | 0 |
| .... | 0.099 | 0.104 | + 5 | 0.011 | 0.011 | 0 |
| 500,000 | 0.172 | 0.172 | 0 | 0.017 | 0.014 | −18 |
| RNase and DNase activity of irradiated homogenates | | | | | | |
| 500,000 | 0.127 | 0.054 | −57 | 0.015 | 0.007 | −52 |
| .... | 0.144 | 0.082 | −43 | | | |
| .... | 0.151 | 0.078 | −48 | | | |

tection. Sodium pyruvate ($1.0 \times 10^{-2}$ *M*), which is rapidly oxidized by Tetrahymena (Seaman, 1949), gave marked protection also.

It is to be noted that L-cysteine (final concentration $3.0 \times 10^{-3}$ *M*) stimulated the respiration of normal cells by approximately 35 per cent, while glutathione in equal concentration increased the oxygen consumption by 15 per cent. It is possible that the tripeptide was hydrolyzed, liberating cysteine which was then oxidized. L-methionine ($2.2 \times 10^{-3}$ *M*) and DL-serine ($6.0 \times 10^{-3}$ *M*) augmented the respiration of normal cells slightly, while glycine ($1.0 \times 10^{-3}$ *M*) and DL-homocysteine ($3.0 \times 10^{-3}$ *M*) had no effect. No auto-oxidation of either L-cysteine or glutathione was observed under the test conditions.

TABLE IV

*The effect of L-cysteine on RNase activity of homogenate irradiated at 500,000 r*

| Experimental conditions | Specific activity | Inhibition (per cent) |
|---|---|---|
| Control | 0.144 | —— |
| X-irradiated | 0.082 | 44 |
| Control + L-cysteine | 0.156 | 0 |
| X-irradiated + L-cysteine | 0.156 | 0 |

*Nuclease determinations.* It is apparent from the data presented in Table III that the activities of both RNase and DNase were little changed in homogenates obtained from whole cells immediately after irradiation at 300,000 and 500,000 r. However, when homogenates were prepared from normal organisms and then irradiated at 500,000 r, both enzymes were markedly inhibited. Irradiation of normal homogenates at 500,000 r in the presence of $3.6 \times 10^{-2}$ *M* L-cysteine completely protected the RNase (Table IV).

TABLE V

*The effect of p-chloromercuribenzoate on RNase activity of homogenates irradiated at 500,000 r*
*See text for description of experiment*

| Experimental conditions | Specific activity | Inhibition (per cent) |
|---|---|---|
| 1. Control | 0.151 | — |
| 2. X-irradiated | 0.078 | 48 |
| 3. Control + p-chloromercuribenzoate | 0.118 | 25 |
| 4. X-irradiated + p-chloromercuribenzoate | 0.036 | 76 |
| 5. X-irradiated + p-chloromercuribenzoate + L-cysteine | 0.078 | 48 |

In the light of the importance placed on the relationship between SH-dependent enzymes and *in vitro* inhibition due to ionizing radiations (Barron *et al.*, 1948–49), it seemed desirable to determine whether the RNase obtained from *T. geleii* W was an SH-dependent enzyme. The homogenate was divided into five 2-ml. portions. Before irradiating with 500,000 r, sample 2 received 2 ml. of distilled water and samples 4 and 5, 2 ml. each of sodium p-chloromercuribenzoate (final concentration $2.6 \times 10^{-3}$ $M$). Samples 1 and 3 were controls without and with p-chloromercuribenzoate. All samples were adjusted to the same pH. Three ml. of each of these solutions were used in the RNase assay which was standard except that 13.4 mg. of L-cysteine were added to the system prepared from sample 5. From the data presented in Table V, it can be seen that the addition of p-chloromercuribenzoate to unirradiated homogenate inhibited the enzyme by 25 per cent. RNase inhibition due to the presence of the SH-binding agent and the effect of x-irradiation was additive, the sum amounting to 76 per cent. The addition of L-cysteine to a mixture of homogenate and p-chloromercuribenzoate immediately after irradiation reversed the effect of the SH-reactant completely but did not alter the inhibition due to radiation.

## DISCUSSION

The question of whether the decreased oxygen consumption exhibited by irradiated Tetrahymena is a reflection of the inhibition of oxidative enzymes, mortality, or both must await further investigation. At 500,000 r, mortality is probably a significant factor. Elliott and Slater (1951) have reported that Tetrahymena were killed at 550,000 r. We have observed repeatedly that immediately after irradiating a solution containing $2 \times 10^6$ cells per ml. with 500,000 r, only an occasional organism exhibited motility; within 24 hours more of the cells regained the ability to move. However, in the light of only a 40 per cent reduction in respiration at 500,000 r, these observations may imply that many of the non-motile cells were alive and their respiratory processes inhibited. The protection against respiratory inhibition which was afforded the organisms by irradiating them in the presence of cysteine and homocysteine is another illustration of the ability of thiol compounds to antagonize the effects of ionizing radiations. The protection of RNase against x-ray inactivation by the addition of cysteine to homogenates is probably a similar phenomenon. This finding is in good agreement with the report of Holmes (1950) who showed that x-ray inactivation of crystalline pancreatic RNase in dilute aqueous solution could be prevented by glutathione. The protective action exerted by sodium pyruvate against respiratory inhibition is interesting in view of the finding that pyruvate will protect bacterial cells against the lethal action of x-rays and $H_2O_2$ (Thompson, Mefferd and Wyss, 1951; Hollaender, Stapleton and Burnett, 1951).

The significant difference observed between the *in vitro* and *in vivo* action of x-radiation on the activities of RNase and DNase is also of considerable interest. Since the intact Tetrahymena occupy roughly 5 per cent of the volume of the irradiated solutions, it may be that 95 per cent of the radio-decomposition products formed in the extracellular water are excluded by the cell membranes. Also, the oxygen tension within the cells is presumably lower than in the external water; thus fewer free radicals would be produced *in vivo*. Rupture of the cell membranes by homogenization would expose the cellular enzymes to the action of the entire quantity of injurious products formed.

From the inhibition of RNase by p-chloromercuribenzoate, it would seem that in Tetrahymena this enzyme is SH-dependent. Crystalline pancreatic RNase (calf) has been shown to be inhibited by several SH-compounds (Zittle, 1946).

The authors wish to acknowledge the assistance of Mr. Stanley Yarus during the course of this work.

## SUMMARY

1. The respiration of x-irradiated *Tetrahymena geleii* W decreased 30 per cent at 300,000 r and 40 per cent at 500,000 r. The addition of cysteine to the organisms prior to irradiation at 300,000 r afforded approximately 50 per cent protection as measured by oxygen consumption. Homocysteine and pyruvate also gave marked protection while methionine had no effect. Cysteine and glutathione stimulated the respiration of normal cells appreciably, but methionine, serine, glycine and homocysteine had little or no effect.

2. Ribonuclease and desoxyribonuclease activities were not changed significantly in homogenates obtained from whole cells which were irradiated at 300,000 and 500,000 r. However, both enzymes were inhibited by about 50 per cent in homogenates which were irradiated at 500,000 r. For RNase, this inhibition was completely reversed by irradiating in the presence of cysteine. Inhibition of RNase of normal Tetrahymena homogenate by p-chloromercuribenzoate was reversed by cysteine.

## LITERATURE CITED

Barron, E. S. G., S. Dickman, J. A. Muntz and T. P. Singer, 1948–49. Studies on the mechanism of action of ionizing radiations. I. Inhibition of enzymes by x-rays. *J. Gen. Physiol.*, **32**: 537–552.

Barron, E. S. G., B. Gasvoda and V. Flood, 1949a. Studies on the mechanism of action of ionizing radiations. IV. Effect of x-ray irradiation on the respiration of sea urchin sperm and eggs. *Biol. Bull.*, **97**: 44–50.

Barron, E. S. G., V. Flood and B. Gasvoda, 1949b. Studies on the mechanism of action of ionizing radiations. V. The effect of hydrogen peroxide and of x-ray irradiated sea water on the respiration of sea urchin sperm and eggs. *Biol. Bull.*, **97**: 51–56.

Billen, D., G. E. Stapleton and A. Hollaender, 1952. The effect of x-radiation on the respiration of bacterial cells. *Nuclear Sci. Abstracts,* **6**: 137.

Chapman, W. H., and E. P. Cronkite, 1950. Further studies of the beneficial effect of glutathione on x-irradiated mice. *Proc. Soc. Exper. Biol. Med.*, **75**: 318–322.

Elliott, A. M., and J. V. Slater, 1951. Action of radiations on survival of Tetrahymena. *Proc. Amer. Soc. Protozool.*, **2**: 8.

Forssberg, A., 1950. On the possibility of protecting the living organism against roentgen rays by chemical means. *Acta Radiol.*, **33**: 296–304.

Hollaender, A., G. E. Stapleton and W. T. Burnett, Jr., 1951. The modification of x-ray sensitivity by chemicals. Isotopes in biochemistry, p. 96. Blakiston Co., Phila.

Hollaender, A., G. E. Stapleton and F. L. Martin, 1951. X-ray sensitivity of *E. coli* as modified by oxygen tension. *Nature*, **167**: 103–104.

Holmes, B., 1950. Inactivation of ribonuclease in dilute aqueous solutions. *Nature,* **165**: 266–267.

McCarty, M., 1948. The occurrence of nucleases in culture filtrates of group A hemolytic streptococci. *J. Exper. Med.*, **88**: 181–188.

Patt, H. M., D. E. Smith, E. B. Tyree and R. L. Straube, 1950. Further studies on modification of sensitivity to x-ray by cysteine. *Proc. Soc. Exper. Biol. Med.,* **73**: 18–21.

Roth, J. S., H. J. Eichel, A. Wase, C. Alper and M. J. Boyd, 1953. Effect of total body x-irradiation on some enzymes of rat tissues. *Arch. Biochem. and Biophys.* (In press.)

Seaman, G. R., 1949. The presence of the tricarboxylic acid cycle in the ciliate *Colpidium campylum*. *Biol. Bull.,* **96**: 257–262.

Seaman, G. R., 1951. Enzyme systems in *Tetrahymena geleii* S. 1. Anaerobic dehydrogenases concerned with carbohydrate oxidation. *J. Gen. Physiol.,* **34**: 775–783.

Smith, D. E., H. M. Patt, E. B. Tyree and R. L. Straube, 1950. Quantitative aspects of the protective action of cysteine against x-irradiation. *Proc. Soc. Exper. Biol. Med.,* **73**: 198–200.

Thompson, T. L., R. B. Mefferd and O. Wyss, 1951. The protection of bacteria by pyruvate against radiation effects. *J. Bact.,* **62**: 39–43.

Wichterman, R., 1948. The biological effects of x-rays on mating types and conjugation of *Paramecium bursaria*. *Biol. Bull.,* **94**: 113–127.

Zittle, C. A., 1946. Ribonucleinase. III. The behavior of copper (Cu) and calcium (Ca) in the purification of nucleic acid and the effect of these and other reagents on the activity of ribonucleinase. *J. Biol. Chem.,* **163**: 111–117.

# STUDIES ON THE DISTRIBUTION OF VITAMIN $B_T$ (CARNITINE)

G. FRAENKEL

*Department of Entomology, University of Illinois, Urbana, Illinois*

The effect and isolation of a new vitamin of the B-complex, $B_T$, which is required by the mealworm and certain other beetle larvae, and its identification as carnitine has previously been reported (Fraenkel *et al.,* 1948; Fraenkel, 1951a, 1951b; Carter *et al.,* 1952). It was stated that $B_T$ had a wide distribution in biological materials, that materials of animal origin were better sources than vegetable matter, and that the muscles of mammals constituted by far the richest known source. Carnitine was originally discovered in commercial meat extracts in which it occurs in amounts of one to two per cent, and was not known to be a general constituent of living systems. Heretofore, the only available method of assay was by isolation. Thus it was impossible to assay for carnitine in substances which contain it in quantities smaller than that found in muscle. With the development of a much more sensitive testing method, using growth and survival of *Tenebrio* as the criterion (Fraenkel, 1951a), it has now become possible to assay for carnitine in a great variety of biological materials.

The dietary need for carnitine has so far been demonstrated only for three beetle larvae, the yellow mealworm, *Tenebrio molitor,* the black mealworm, *Tenebrio obscurus,* and another related species, *Palorus ratzeburgi.* The present paper concerns the levels of carnitine in a great variety of sources, animal and vegetable, and also gives evidence of a synthesis of carnitine in organisms which can develop in the absence of carnitine. Throughout this paper the terms "carnitine" and "$B_T$" will be used almost synonymously; the possibility exists, however, that carnitine may not be the only naturally occurring substance with $B_T$ activity. We are, therefore, assaying, strictly speaking, for $B_T$ activity and not for carnitine.

## Methods of Assay and Tabulation

The methods of assaying and expressing results have been previously described in detail (Fraenkel *et al.,* 1950; 1951a). Briefly, *Tenebrio* larvae grow slowly and begin to die after 3–4 weeks on a "synthetic" diet consisting of 20 parts casein, 80 parts glucose, one part cholesterol, two parts McCollum's salt mixture No. 185, 10 parts water and the following vitamins of the B-complex (expressed as $\mu$g./g. of the dry diet): thiamin 25, riboflavin 12.5, nicotinic acid 50, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, folic acid 2.5 and biotin 0.25. When a good source of $B_T$ or 0.35 $\mu$g./g. carnitine is added to the "basic" diet, the larvae grow at a steady rate and survive.

Table I shows the result of an experiment with graded doses of carnitine. It can be seen that survival is optimal at levels of 0.375 $\mu$g./g., while the weights still increase at levels of 0.75 and 1.50 $\mu$g. Growth and survival at the 50 $\mu$g. level are virtually identical with that at 1.5 $\mu$g. In all our determinations we have followed a procedure whereby, in a series of graded doses, that diet which allows optimal survival and good, but not necessarily optimal growth, is assumed to contain 0.35 μg. carnitine/g. dry diet.

With levels of 0.35 μg. carnitine per gram, larvae reach an average weight of about 60 mg. after 9–10 weeks of growth at 29–30 ° C. and 60% relative humidity This level of carnitine is sufficient to maintain normal growth only up to this point. Subsequently, growth gradually slows down and the larvae may develop signs of a $B_T$ deficiency. In order to reach a normal weight of 140 mg., after a period of 13 weeks, and to pupate and emerge as normal adults, a level of 1.5 μg. carnitine per gm. diet is required (unpublished data). To base assays on optimal growth and development to the adult stage, experiments would have to be continued for 25 weeks and would also require two to three times as much food. In order to economize in time and food consumption, the assays were all based on the 9 weeks-60 mg. criterion.

TABLE I

*Numbers surviving and average weights of Tenebrio larvae on the basic diet and with the addition of yeast or graded doses of carnitine. Twenty larvae were used in each of the control diets and 60 larvae with each concentration of carnitine; at 30° C. and 60% relative humidity*

| Weeks | Control diets | | | | μg. carnitine/g. of the dry diet | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No addition | | 2% yeast | | 0.19 | | 0.375 | | 0.75 | | 1.5 | | 50 | |
| | Nos. | Mg. | Nos. | Mg. | Nos. | Mg. | Nos. | Mg. | Nos. | Mg. | Nos. | Mg. | Nos. | Mg. |
| 5 | 9 | 7.4 | 17 | 9.5 | 45 | 8.3 | 48 | 8.9 | 48 | 10.7 | 50 | 10.0 | 50 | 10.2 |
| 6 | 6 | 9.7 | 17 | 15.0 | 42 | 13.3 | 48 | 14.9 | 46 | 19.3 | 49 | 19.1 | 50 | 19.4 |
| 7 | 5 | 12.2 | 16 | 23.5 | 40 | 21.3 | 47 | 22.3 | 46 | 29.6 | 47 | 29.8 | 50 | 29.2 |
| 8 | 4 | 13.2 | 15 | 31.4 | 38 | 24.0 | 47 | 31.8 | 46 | 42.2 | 47 | 41.0 | 48 | 45.6 |
| 9 | 4 | 16.2 | 15 | 39.7 | 36 | 34.6 | 47 | 42.5 | 45 | 59.5 | 47 | 57.6 | 48 | 59.2 |
| 10 | 3 | 24.3 | 15 | 50.7 | 34 | 47.5 | 46 | 56.0 | 45 | 64.6 | 47 | 72.5 | 48 | 74.6 |

In assaying different materials for a particular substance, it is important to consider the form in which they exist in the diet. Only finely ground powders, such as flour, dried egg or dried milk, can be directly mixed into the diet in the dry state. In most cases, the materials have to be either homogenized in water, with the use of the Waring Blendor, or Potter-Elvehjem homogenizer, or else prepared as extracts. All such materials are then pipetted into the diets in the required amounts and dilutions. To avoid losses in the extraction process, it is always preferable to use homogenates. The Potter grinder homogenizes soft tissues well, and more resistant tissues can often be homogenized with the Waring Blendor. Wherever a tissue did not lend itself to homogenation suitable for pipetting, extracts had to be prepared. In such cases, tissues were first homogenized or ground in a mortar and then extracted with several portions of boiling water for periods of 30 minutes each, and the combined extracts concentrated to the desired volume. In some cases alcohol was added to aqueous extracts to make a concentration of 75 to 80%. The ensuing precipitates were removed by centrifugation and the alcohol evaporated off over a water bath.

In expressing results, a different procedure was followed with powdered materials or whole homogenates, than with extracts. In the former cases, amounts are expressed as percentages of the dry materials, added to the diet. With extracts, levels are often calculated as percentages of the dry, or in rare instances, wet materials from which the extracts were derived.

## EXPERIMENTS

### *1. The $B_T$ content of microorganisms (Table II)*

The effect of $B_T$ was originally discovered in experiments with *Tenebrio* larvae which failed to grow on synthetic diets, but which grew well after two per cent yeast had been added. The $B_T$ content of yeast seems to vary in different preparations. Two per cent dry brewers yeast in the diet, the level routinely used in our control experiments, usually ensures optimal survival, but the larvae may still be somewhat underweight (Table I). The $B_T$ activity of three different strains of brewers yeast, obtained from Anheuser-Busch, varied between 1% and 4%. The $B_T$ content of *Torula* yeast and of *Neurospora* was of the same order as that of brewers yeast.

TABLE II

*The $B_T$ content of several microorganisms and materials of vegetable origin*

| | Minimum amount to give full activity | Maximum amounts tested and found inactive | $B_T$ μg./g. solids | Description of preparation |
|---|---|---|---|---|
| Microorganisms: | | | | |
| *Escherichia coli* I[1] | | 5% | <1* | Grown on synthetic medium |
| *Escherichia coli* II[1] | | 5% | <1* | Grown on synthetic medium |
| *Escherichia coli* III[2] | | 5% | <1* | Grown on synthetic medium |
| *Streptococcus hemolyticus*[2] | 1.75% | | 28 | Medium not entirely synthetic |
| Torula yeast | 1–2% | | 17.5–35 | |
| Brewers yeast I | 2–4% | | 8.8–17.5 | Anheuser-Busch |
| Brewers yeast II | 1% | | 35 | Anheuser-Busch |
| Brewers yeast III | 1% | | 35 | Anheuser-Busch |
| Brewers yeast IV | 1–2% | | 17.5–35 | Nutritional Biochemicals |
| Neurospora[3] | 1.25 | | 28 | Grown on synthetic medium |
| *Tetrahymena geleii*[4] | | 5% | <1* | Grown on synthetic medium |
| Vegetable matter: | | | | |
| Wheat | 2.5–5% | | 7–14 | |
| Corn | | 10% | <0.5* | |
| Alfalfa concentrate[5] | 1.8% | | 20 | Spray-dried |
| Rye juice[5] | | 1.8% | <2* | Spray-dried |
| Oats juice[5] | | 1.8% | <2* | Spray-dried |

Prepared by: [1] I. Gunsalus, [2] Sharp & Dohme, [3] E. L. Tatum, [4] G. W. Kidder, [5] Cerophyl Laboratories.

* Possibly none.

Two of the microorganisms tested, *Escherichia coli* and *Tetrahymena geleii,* were entirely devoid of $B_T$ activity when included in the diet in levels up to 5%. Full activity at this level would have indicated a carnitine content of 7 μg./g., and there would have been an indication of activity had the carnitine content been only 1 μg./g. It would be difficult to test for such small quantities, because of the large proportion, 40% and more, of assay material, which would have to be included in the diet. Both these organisms were cultured in synthetic media which in all probability were free of $B_T$. We may, therefore, conclude that these two organisms neither require nor synthesize carnitine, or synthesize it in amounts below the sensitivity of our assay method. On the other hand, *Streptococcus hemolyticus* from a medium which was not entirely synthetic showed $B_T$ activity at the 1.25% level.

### 2. *The $B_T$ content of vegetable matter* (*Table II*)

It was previously shown (Fraenkel, 1951a) that coconut meal, peanut meal and cotton seed meal failed to show any $B_T$ activity in concentrations up to 3% of the diet. The $B_T$ content of all vegetable matter which has so far been tested was low. Of cereal seeds, wheat showed good activity in concentrations of 2.5 to 5%, but corn was inactive in amounts up to 10%. Of a few samples of juices which had been pressed out of young plants and spray-dried, alfalfa juice was active at a level of 1.8%, while rye and oat juice failed to show any activity at the same level.

### 3. *$B_T$ requirements and $B_T$ contents of insects*

A dietary requirement for carnitine has so far been demonstrated for three insect species only, *Tenebrio molitor, Tenebrio obscurus* and *Palorus ratzeburgi.* The larvae of at least 15 other species of insects have been successfully grown on basic diets, similar to that used for *Tenebrio,* in the absence of carnitine. The question then arose, whether carnitine was present in the tissues of insects grown in its absence. It would also be of interest to know to what extent the amount of carnitine present in the tissues of *Tenebrio* larvae corresponded to the levels which had been fed in the diet.

#### *a. Carnitine content of Tenebrio larvae*

1. *Larvae grown largely in the absence of carnitine.* These larvae had been grown for the first three weeks on the deficient basic diet, then for 10 days on a normal optimal diet (whole meal plus 5% yeast) and subsequently again on the synthetic deficient diet. They continued to grow for a period of about 7 weeks until they reached a weight of 40–60 mg., then stopped growing and ultimately died. Extracts from these deficient larvae, incorporated in diets in amounts corresponding up to 4% larval bodies, failed to show any $B_T$ activity.

2. *Larvae grown on an optimal diet, consisting of wholemeal flour plus 5% yeast* (Table III). Extracts from these larvae, incorporated in diets in quantities corresponding to 1% of dry larval bodies, gave good growth and almost optimal survival. The result indicated that 2% would have ensured optimal survival.

One gram of wheat flour contains 7 μg. $B_T$ (Table II) and the addition of 5% yeast (containing 17.5 $B_T$/g.) only insignificantly increases that amount. The growing *Tenebrio* larva eats food in amounts of about 4.5 times its ultimate dry body weight. Thus a nearly full-grown larva, which weighs about 100 mg. (40 mg. dry weight), has consumed 180 mg. of food, containing 1.3 μg. of carnitine. Therefore, 32.5 μg. carnitine are consumed per gram dry weight of larvae. If all this carnitine were retained in the body of the larva, a diet with 2% dried larvae added as the sole source of carnitine would contain 0.65 μg. carnitine per gram. However, larvae grown on this particular diet only showed optimal activity. Since 0.35 μg. $B_T$ is the minimal amount required for optimal activity, it may be assumed that the diet actually contained only 0.35 μg. carnitine per gram. Thus the actual carnitine content of the dried larvae would be 17.5 μg./g. The growing larvae therefore retained almost half the carnitine which they took up in the food.

3. *Larvae grown on graded levels of carnitine.* In one experiment, extracts from *Tenebrio* larvae, which had been grown on diets containing 0.37, 0.75 and 1.5 μg. $B_T$ per gram, were incorporated in diets and tested in quantities corresponding up to 4% of the diets. None of these diets showed $B_T$ activity. It was calculated that even with 1.5 μg. in the diet, and even if all the carnitine had been retained in the larvae, only 0.27 μg. carnitine per gram could have been present in diets containing 4% of dried larval bodies.

TABLE III

*The $B_T$ content of two beetle larvae, Dermestes vulpinus and Tenebrio molitor, grown in the presence and absence of $B_T$. Tested as aqueous extracts in their effect on growth and survival of Tenebrio larvae after 9 weeks*

| | Concentration in diet corresponding to | Grown in the | | | |
|---|---|---|---|---|---|
| | | Presence of $B_T$ | | Absence of $B_T$ | |
| | | Nos. | Mg. | Nos. | Mg. |
| *Dermestes vulpinus* | 0.125% | 8 | 66.1 | 10 | 55.2 |
| | 0.25% | 15 | 72.2 | 7 | 71.2 |
| | 0.5% | 13 | 67.7 | 13 | 77.6 |
| | 1.0% | 12 | 89.6 | 14 | 85.7 |
| | Controls: no $B_T$ | 3 | 32.6 | | |
| | 2% yeast | 18 | 74.6 | | |
| *Tenebrio molitor* | 0.125% | | | 2 | 47.0 |
| | 0.25% | 6 | 43.7 | 1 | 4.5 |
| | 0.5% | 6 | 61.6 | 0 | — |
| | 1.0% | 12 | 53.0 | 0 | — |
| | Controls: no $B_T$ | 0 | — | | |
| | 2% yeast | 18 | 96.1 | | |

Calculated $B_T$ contents (μg./g. dry insect bodies)
grown in presence of $B_T$: *Tenebrio* 17.5; *Dermestes* 140
grown in absence of $B_T$: *Tenebrio* none; *Dermestes* 70–140

In a subsequent experiment the $B_T$ content was determined of larvae which had been grown on synthetic diets containing 1.5 μg. and 50 μg. carnitine/g. respectively. Extracts from larvae, grown on 1.5 μg. carnitine per gram of food, gave an optimal response in quantities corresponding to 7.5% dry larval bodies; and extracts from larvae grown on 50 μg. carnitine/g. showed optimal activity in quantities corresponding to 0.3 to 0.6% larval bodies. Making use of these figures it is calculated that 7.5% of larval tissue, grown at a level of 1.5 μg. $B_T$/g., would have provided 0.5 μg. carnitine/gm. diet, if all the carnitine fed to the larvae had been retained. Correspondingly, 0.3–0.6% of larvae raised on a diet with 50 μg./g. $B_T$ could have provided a maximum of 0.35 to 0.7 μg. $B_T$/g. Since the diet at the levels stated only showed optimal activity which is assumed to correspond to a level of 0.35 μg. $B_T$/g., it is obvious that in this experiment almost all the carnitine which the larvae had eaten was retained (Table IV).

The $B_T$ contents of larvae grown on diets containing 1.5 and 50 μg. $B_T$/g. were calculated as 5 μg. and 56 to 112 μg./g., respectively.

In a further experiment, larvae were raised on whole meal flour plus 5% yeast until they reached a body weight of 20 mg. live weight. One portion of these larvae was tested for $B_T$, and another transferred to a basic carnitine-free diet and tested when the average weight reached 105 mg. The 20-mg. larvae showed good activity at levels of 1.25–2.5% which correspond to a carnitine content of 14–28 μg./g. This is in good agreement with the results of a similar assay referred to above (Table IV). The other larvae, after having reached a weight of 105 gm., showed no carnitine activity in levels up to 7.5% of the diet. If all the carnitine which was present in the 20-mg. larvae had been retained, the 105-mg. larvae would have been expected to have 5 times less carnitine per unit weight, and would have shown carnitine activity at levels of 6–12%. The fact that no activity was shown at the 7.5% level indicates some loss of carnitine during growth from 20 to 105 mg.

TABLE IV

*Relations between $B_T$ content of the food, the retention of $B_T$ in Tenebrio molitor larvae and the $B_T$ content of larvae grown on these foods*

| | Nature and $B_T$ content of diets of *Tenebrio* larvae to be assayed for their $B_T$ content | | |
|---|---|---|---|
| | Whole meal flour + 5% yeast 7 μg. $B_T$/g. | Synthetic diet 1.5 μg. $B_T$/g. | Synthetic diet 50 μg. $B_T$/g. |
| Smallest quantity of *Tenebrio* tissue to give optimal $B_T$ effect | 2% | 7.5% | 0.3–0.6% |
| Amount $B_T$ which would have been present in test diets if all $B_T$ taken up had been retained | 0.65 μg./g. diet | 0.5 μg./g. diet | 0.35–0.70 μg./g. |
| Amount $B_T$ found by bio-assay | 0.35 μg./g. diet | 0.35 μg./g diet | 0.35 μg./g. diet |
| Calculated $B_T$ content of larvae | 17.5 μg./g. tissue | 5 μg./g. tissue | 56–112 μg./g. tissue |

All these tests with *Tenebrio* indicate clearly:

1. That no synthesis of carnitine takes place in *Tenebrio* larvae, and
2. that growing *Tenebrio* larvae retain 50% or more of the carnitine which they take up with the food.

### *b. The carnitine content of the larvae of Dermestes vulpinus*

The larder beetle, *Dermestes vulpinus,* has been raised successfully on a diet consisting of 80 parts casein, 20 parts fructose, one part cholesterol, two parts

McCollum's salt mixture and the mixture of 8 vitamins, as described above (Fraenkel, 1953). A comparison of the carnitine contents of *Dermestes* grown on this diet in the absence of carnitine, or on a mixture of 95% fish meal and 5% yeast, which contains high amounts of carnitine (Table VIII), showed them to be virtually the same (Table III). In both cases *Dermestes* bodies gave good activity in the diet at levels of 0.25 to 0.5%, which corresponds to a carnitine content of 70 to 140 μg./g. Carnitine is therefore synthesized with great efficiency in the *Dermestes* larva.

### *c. The carnitine content of the larvae of the blowfly, Phormia regina*

The larvae of *Phormia regina,* grown on a diet of raw liver, under nonaseptic conditions, showed full $B_T$ activity when incorporated in the diet at a level of 0.062% which corresponds to the very high content of 560 μg. carnitine/gm. (dry). Larvae grown on a synthetic diet in the absence of carnitine under sterile conditions showed full activity in a *Tenebrio* diet in levels of 2%. This corresponds to a carnitine content of 17.5 μg./g. or about 30 times less than the amount found in larvae grown on liver. Carnitine is therefore also synthesized in the fly larva, but at a lower level than in *Dermestes.*

In another test, the carnitine content of fly larvae which had been grown on liver was followed from the fully grown larva through the pupal stage to the young adult. Fully grown larvae, two-, three-, and four-day old pupae and adults one day after emergence were tested. The carnitine content was the same in all stages, about 560 μg./g. dry weight. Since synthesis of carnitine in the fly larva is relatively slow, we may assume that the bulk of the carnitine, which has accumulated during larval development, is retained in the pupa and later incorporated in the tissues of the adult fly.

In summary we may therefore state that synthesis of carnitine takes place in two insect species (*Dermestes* and *Phormia*) which do not require it in the diet, while *Tenebrio,* which requires it in the diet, shows no signs of synthesis under any conditions.

### *4. The $B_T$ content of eggs, chick embryos and chicks*

The hen's egg had previously been shown to contain surprisingly little $B_T$. A test with dried egg powder suggested full activity at a level of 6% in the diet (Fraenkel, 1951a). Subsequently several tests with raw homogenized egg showed an even lower activity. Three per cent (on a dry weight basis) was entirely inactive and could hardly have shown optimal activity at less than a 12% level. It was then thought that the negative results obtained with raw egg white might have been caused by a toxic egg-white factor. This assumption proved to be incorrect. Extracts made from boiled egg were only a little more active than the corresponding amount of raw egg. Finally, pure carnitine, at levels of 1.5 μg./g. diet, was added to diets which contained raw or boiled egg in concentrations up to 3 per cent. Growth was uniformly optimal in all tests with added $B_T$. This showed conclusively that the negative results with raw egg could not have been due to the presence of a toxic factor.

Subsequently, it was found that the hatching chick contained demonstrable amounts of $B_T$. This suggested the synthesis of $B_T$ in the growing chick embryo.

A test was then designed in which eggs (single comb White Leghorn) were incubated, and the embryos and remaining yolk and white portions analyzed for $B_T$ after periods of 8, 12, 16 and 20 days, and in the one-day old unfed chick. The embryos were dissected out and, after removal of the membranes, homogenized in a Potter-Elvehjem homogenizer (8- and 12-day embryos) or the Waring Blendor (16- and 20-day embryos). The results are shown in Table V. All the embryos showed about the same carnitine activity, with optimal effects at levels of 0.5%. No $B_T$ activity was shown in the remaining white and yolk fractions at levels up to 3%, in the 8-, 12- and 16-day egg, and a low activity, at a level of 2.3%, in the 20-day egg. The $B_T$ activity of the whole one-day chick could not be measured accurately, but appeared to be of the same order as that of the embryos. However, tests with three tissues, brain, liver and muscle, showed $B_T$ activities of an order similar to those encountered in several mammals (Table VI).

TABLE V

*The $B_T$ activity of egg and of chick embryo and other egg fractions after varying periods of incubation. Tested as whole homogenized preparations (with the exception of muscle—one-day chick) in their effect on growth and survival of Tenebrio larvae after 9 weeks*

| Period of incubation | Weight of embryo or chick | Material | Minimum amount to give full activity | Maximum amounts tested found inactive | Calculated $B_T$ contents μg./g. dry tissue |
|---|---|---|---|---|---|
| 0 days | | Whole egg | | 3% | <3 |
| 8 days | 0.9 g. | Yolk + white | | 2.8% | <3 |
| | | Embryo | 0.35–0.7% | | 50–100 |
| 12 days | 4 g. | Yolk + white | | 2.8% | <3 |
| | | Embryo | 0.4–0.8% | | 44–88 |
| 16 days | 14 g. | Yolk + white | | 3% | <3 |
| | | Embryo | 0.5% | | 70 |
| 20 days | 31 g. | Yolk + white | 2.3% | | 15 |
| | | Embryo | 0.4% | | 88 |
| One-day chick | 40 g. | Brain | 0.5–1% | | 35–70 |
| | | Muscle extract | 0.075% | | 466 |
| | | Liver | 0.56% | | 70 |

If we assume a water content of the 20-day (31 g.) embryo of 80%, and an optimal $B_T$ activity at the 0.5% level, this embryo would have contained 420 μg. carnitine and the egg less than 52.5 μg., assuming an egg of 50 g. (15 gm. dry weight) and optimal $B_T$ activity with more than 10% egg in the diet. It is evident that nearly all the carnitine in the chick must have arisen from synthesis.

### *5. The $B_T$ content of mammalian tissues, blood and urine*

It has been previously shown that various organs of mammals are rich sources of $B_T$ (Fraenkel, 1951a). These determinations were carried out with fat-free powders. Subsequently it was found that the mammalian skeletal muscle showed three to five times higher $B_T$ activity than the best tissue preparations previously tested. More assays have now been carried out using homogenates and extracts from fresh organs from the dog, rat and rabbit (Table VI). With tissue homogenates, prepared with the aid of the Waring Blendor or Potter-Elvehjem homogenizer, the calculations in Table VI are based on the solid content of the homogenates. In the case of extracts, the water content of tissues from which the extracts were prepared was not determined at the time. For these calculations a water content of 70% was assumed, which introduces small errors.

The results obtained from fresh dog tissues were of a similar order as those previously determined from tissue powders (Table VI). Brain and nerve contained relatively small amounts of $B_T$, and the $B_T$ content of blood, on a dry weight basis, was also low, for an animal tissue. The highest concentrations of $B_T$ were again found in muscle, with the skeletal muscle highest, a smooth muscle (bladder) lowest, and the heart muscle intermediate. The $B_T$ content of muscle and liver from rabbit and rat differed only slightly from those of the dog.

TABLE VI

*$B_T$ (carnitine) content of various mammalian tissues, expressed as μg./g. dry tissues*

| Organism | Tissue | Minimal conc. for optimal activity % | $B_T$ μg./g. dry tissue | Method |
|---|---|---|---|---|
| Dog I | Muscle, leg | 0.03 | 1120 | Homogenate |
| | Liver | 0.12 | 280 | Homogenate |
| | Brain | 0.4 | 87 | Homogenate |
| | Pancreas | 0.33 | 105 | Homogenate |
| | Small intestine | 0.15 | 224 | Homogenate |
| | Blood | 1.0 | 35 | Whole |
| Dog II | Muscle, leg (normal) | 0.03 | 1120 | Extract |
| | Muscle, leg (paralyzed) | 0.03–0.06 | 560–1120 | Extract |
| | Heart | 0.06 | 560 | Extract |
| | Bladder | 0.06–0.12 | 280–560 | Extract |
| | Nerve | 0.25–0.5 | 70–140 | Homogenate |
| Dog III | Liver | 0.25 | 140 | Homogenate |
| | Kidney | 0.085 | 412 | Homogenate |
| | Liver | 0.5 | 70 | Extract |
| | Liver, autolysed | 0.25–0.5 | 70–140 | Homogenate |
| | Liver, autolysed | 0.5 | 70 | Extract from autolysed liver |
| Rat | Muscle, leg, laboratory diet | 0.03–0.06 | 560–1120 | Homogenate |
| | Muscle, leg, $B_T$-free diet | 0.05–0.1 | 350–700 | Homogenate |
| | Liver, laboratory diet | 0.17–0.34 | 100–200 | Homogenate |
| | Liver, $B_T$-free diet | 0.15–0.3 | 112–224 | Homogenate |
| Rabbit | Muscle, leg | 0.048 | 700 | Homogenate |
| | Liver | 0.09 | 370 | Homogenate |

From the high concentration of $B_T$ in skeletal muscle it was assumed that $B_T$ may play some important function in the metabolism of muscle. In order to determine whether or not the concentration of $B_T$ in the skeletal muscle was dependent on its functioning, one hind leg of a dog was paralyzed by severing the nerve leading to it, and corresponding portions of muscle from the paralyzed and normal leg were analyzed for their $B_T$ content three weeks later. The carnitine content of the paralyzed muscle was very slightly, and probably insignificantly, lower than that of the normal muscle (Table VI).

Another comparison was made between the $B_T$ content of liver and muscles from rats fed on a normal laboratory diet, which contained carnitine, and others fed on a synthetic diet, which did not contain it. The results suggested that the carnitine contents of these two tissues were not dependent on the supply in the diet, and that therefore, in all probability, carnitine is synthesized in the rat.

In a further experiment the stability of carnitine to the action of tissue enzymes was investigated. Homogenates of dog liver were subjected to autolysis for three days at 30° C. in the presence of toluene. At the end of the period there was a strong smell of putrefaction in the samples. There was no change in the carnitine content as a result of autolysis and putrefaction.

Table VII contains data concerning the $B_T$ content of urine and blood from human subjects. Here the $B_T$ content is expressed as μg./ml. of urine or blood.

TABLE VII

*$B_T$ (carnitine) content of urine and blood from human subjects*

| Sex and age | $B_T$ μg./ml. | Description |
|---|---|---|
| | | Urine |
| ♂50 | 132 | 24-hour sample |
| Same | 132–264 | 3 hours after heavy meat meal |
| Same | 28–56 | 20 hours on diet low in $B_T$ |
| ♀21 | 56 | High protein diet |
| ♂23 | 56 | High protein diet |
| ♀22 | 14–28 | Diet high in vegetable and fruit |
| ♂24 | 28–56 | Diet high in vegetable and fruit |
| ♀21 | 132 | After 3 days of starvation |
| ♂25 | 132–264 | After 3 days of starvation |
| Students | 28–56 | Pooled sample from about 50 students |
| | | Blood |
| | 7–14 | Pooled, whole blood |
| | 7–14 | Pooled, plasma |

The $B_T$ content of urine showed fairly large variations which seemed to be dependent on the $B_T$ intake in the diet. The highest figures, 132–264 μg./ml., were found after a heavy meat meal and at the end of a starvation period of three days. This, in the case of starvation, may be due to breakdown of muscle substance and concomitant release of $B_T$. The lowest levels, 14–28 μg./ml., were obtained from persons who had been on a vegetable diet low in carnitine.

The $B_T$ content of human blood was uniformly low in several determinations (7–14 μg./ml.) and the activity of whole blood and plasma was the same.

### *6. The $B_T$ activity of various extracts*

In the preceding pages, the carnitine contents of diverse biological materials have been stated in terms of μg. per gram dry weight of that particular material. When assaying extracts, it is also of interest to know the carnitine activity in relation to the solid content of the extracts. Table VIII contains data about the $B_T$ activity of various extracts which were used in the course of this work. With commercial preparations, nothing is known about the procedures of preparation, but it is assumed that they were mainly hot water extracts. In our own preparations, the homogenized samples were extracted with two or three portions of water at 100° C. In some cases, the hot water extracts, after concentration in an evaporating dish, were treated with alcohol, to give a concentration of 75 to 80 per cent, and the ensuing precipitates removed.

Table VIII shows that meat extracts were by far the most active preparations, containing carnitine in amounts from one to almost three per cent. Whey and liver extracts, which were used in the isolation of carnitine (Carter *et al.*, 1952), contained 5 to 10 times less carnitine than muscle extracts. Fish extracts were of a similar order of activity. Brewers yeast, the material on which the $B_T$ effect was originally discovered and which had been used in the early isolation work, proved a very inferior source. A standard preparation of vitamin T (Pharmazell GMBH, Raubling, Obb.) proved to be of the same order as yeast extract. (Vitamin T concentrate has the appearance, smell and taste of yeast extract, and, in all probability, is nothing but a crude yeast extract.)

TABLE VIII

*The $B_T$ activity of various extracts. The activity is expressed as µg. solids per gram of the dry diet (A) and as µg. $B_T$ per gram solids in the extracts (B)*

| | A<br>Minimum amount to give optimum effect µg./g. | B<br>$B_T$ per gram solids µg./gm. | Method of preparation |
|---|---|---|---|
| Beef muscle extract (Wilson) | 36–72 | 4860–9720 | Aqueous extract from dried muscle powder |
| Meat juice (Wilson) | 15–30 | 11,650–23,300 | Commercial preparation, probably aqueous extract |
| Difco beef extract | 12–24 | 14,600–29,200 | Commercial preparation, for bacteriological use |
| Liver extract (Wilson) | 125 | 2800 | Aqueous extract, alcohol treated |
| Heart infusion | 125–250 | 1400–2800 | Commercial preparation |
| Whey extract | 360 | 972 | Aqueous extract from dried whey (Borden), alcohol-treated |
| Fish solubles | 500 | 700 | Commercial preparation |
| Fish meal extract | 100 | 3500 | Aqueous extract |
| Yeast (brewers) extract | 2000 | 175 | Aqueous extract, alcohol treated |
| Vitamin T concentrate | 3400 | 103 | Commercial preparation (Pharmazell GMBH) |

## DISCUSSION

Our investigations concerning distribution and requirements of Vitamin $B_T$ (carnitine) have made it abundantly clear that $B_T$ occurs widely, if not perhaps universally, in all living matter and that animals either require it in the diet or else synthesize it. We have demonstrated the presence of $B_T$ in a great variety of organisms, ranging from yeast to mammals, but have been unable to find it in a bacterium, *Escherichia coli,* a protozoon, *Tetrahymena geleii,* and in corn seeds. It would be rash to conclude from the available data whether or not it is a necessary constituent of all living matter. Our testing method, using growth and survival of an insect, *Tenebrio molitor,* is not sensitive enough to detect amounts below about one μg./gm. of the dry substance. Furthermore, the possibility must be kept in mind that it might occur in some organisms in a form in which it is not available for *Tenebrio.*

At this point we may ask why we would expect carnitine to occur in all living matter. We have seen that it occurs regularly in animals, and that those organisms which do not require it in the diet synthesize it. This, together with the fact that *Tenebrio* requires it in very small quantities, 0.37 to 0.75 μg./g. of the food, that is to say on the catalytical level, would indicate that it functions in a process of vital importance, at least for yeast, higher plants, insects and vertebrates. Processes of this description are known in the functioning of enzyme systems of the intermediary metabolism and are usually characteristic for all living matter.

On the other hand, it is difficult to reconcile the fact, that $B_T$ is required by *Tenebrio* on the level of some of the most potent B vitamins, biotin and folic acid, but occurs in certain tissues, especially muscle, in quantities of one mg./g. This may suggest that it might be involved in two different functions, one in which it acts on the catalytical level, and one in which vastly larger quantities are involved.

Leclerq (1950) stated that the growth rate of *Tenebrio molitor* was greatly increased by the addition of a preparation of vitamin T (Goetsch, 1947) to the synthetic diet and considers the possibility that vitamin T and $B_T$ could be identical. (The similarity in the designation of these two growth factors is entirely accidental.) We have found only weak $B_T$ activity in a vitamin T preparation which entirely excludes this possibility. It has, however, since been shown that "vitamin T," a crude extract from Torula yeast, contains a multitude of factors (Goetsch, 1951; Wacker *et al.,* 1951).

This investigation was supported in part by research grants from the National Institutes of Health, Public Health Service, and the Graduate College Research funds of the University of Illinois. I gratefully acknowledge the aid of Dr. F. R. Steggerda and Dr. H. M. Scott, University of Illinois, in certain phases of the work; and that of Drs. I. Gunsalus, University of Illinois, E. L. Tatum, Stanford University, and G. K. Kidder, Amherst College; also Cerophyl Laboratories, Sharpe and Dohme, Anheuser-Busch and the Wilson Company for supplying materials and preparations for testing. It is also a pleasure to acknowledge the careful assistance of Mrs. E. Nilson.

## SUMMARY

1. The procedure in the bio-assay of $B_T$ (carnitine) with the use of *Tenebrio molitor* as the subject has been described. The minimum requirements of carnitine to give optimal survival and nearly optimal growth of the *Tenebrio* larva are 0.35 μg. per gram of the dry diet. For optimal growth 1.5 μg. per gram are required.

2. Yeast and Neurospora contain about 35 μg. carnitine/g., and wheat 7–14 μg./g. No evidence of the presence of carnitine could be obtained from *Escherichia coli, Tetrahymena geleii* and corn.

3. Synthesis of carnitine takes place in two insect species (*Dermestes* and *Phormia*) which do not require it in the diet, while *Tenebrio*, which requires it in the diet, shows no sign of synthesis under any conditions. The growing *Tenebrio* larva retains more than 50% of the carnitine which it takes up in the diet.

4. Little carnitine was found in the hen's egg. It is synthesized in sizeable quantities in the growing chick embryo.

5. In mammalian tissues, by far the largest quantities of carnitine, about 1000 μg./g. of dry tissue, occur in the skeletal muscle. Corresponding figures for other tissues were: heart 560, bladder 280–560, kidney 412, liver 140–280, small intestine 224, brain 87, nerve 70–140 and blood 35 μg./g. dry tissue.

6. In dog muscle which had been paralyzed for several weeks, the carnitine content was not significantly changed. Liver after autolysis had the same carnitine content as before.

7. The carnitine content of pooled samples of human urine was 28–56 μg./ml. In different individuals levels as low as 14–28 and as high as 132–264 μg./ml. were found. This variation depended on the carnitine content of the diet, with the highest figures after a heavy meat meal or a three-day period of starvation, and the lowest after a vegetable diet. Blood contains fairly uniform levels of 7–14 μg./ml.

8. Different commercial preparations of meat extracts had carnitine contents of one to three per cent (dry weight).

9. Since carnitine occurs universally, or almost universally in biological material, and an organism either synthesizes it, or else requires it as a "vitamin," it is considered to be of vital importance in the metabolism of most or all forms of life.

## LITERATURE CITED

Carter, H. E., P. K. Bhattacharyya, K. R. Weidman and G. Fraenkel, 1952. Chemical studies on vitamin $B_T$. Isolation and characterization as carnitine. *Arch. Biochem. Biophys.*, **38**: 405–416.

Fraenkel, G., 1951a. Effect and distribution of vitamin $B_T$. *Arch. Biochem. Biophys.*, **34**: 457–468.

Fraenkel, G., 1951b. Isolation procedures and certain properties of vitamin $B_T$. *Arch. Biochem. Biophys.*, **34**: 468–477.

Fraenkel, G., 1952. The nutritional requirements of insects for known and unknown vitamins. *Trans. IX. Intern. Congr. Entomology*, **1**: 277–280.

Fraenkel, G., M. Blewett and M. Coles, 1948. $B_T$, a new vitamin of the B-group and its relation to the folic acid group, and other anti-anaemia factors. *Nature*, **161**: 981–983.

Fraenkel, G., M. Blewett and M. Coles, 1950. The nutrition of the mealworm, *Tenebrio molitor*. *Physiol. Zool.*, **23**: 92–108.

Goetsch, W., 1947. Vitamin T, ein neuartiger Wirkstoff. *Österr. zool. Zeitschr.*, **1**: 49–57.

Goetsch, W., 1951. Ergebnisse und Probleme aus dem Gebiet neuer Wirkstoffe. *Österr. zool. Zeitschr.*, **3**: 140–174.

Leclerq, J., 1950. La vitamin T, facteur de croissance des larves de *Tenebrio molitor*. *Arch. Intern. Physiol.*, **57**: 350–352.

Wacker, A., H. Dellweg and E. Rowold, 1951. Biochemical studies with vitamin T. *Klin. Wochenschr.*, **29**: 780–783. (Quoted from *Chem. Abstr.*, **46**: 5155, 1952.)

# BLOOD COAGULATION IN ARTHROPODS. III. REACTIONS OF INSECT HEMOLYMPH TO COAGULATION INHIBITORS OF VERTEBRATE BLOOD[1]

CH. GRÉGOIRE

*Department of Biochemistry, University of Liège, Institut Léon Fredericq,*
*17 Place Delcour, Liège, Belgium*

As shown in previous studies by means of the phase contrast microscope (Grégoire and Florkin, 1950; Grégoire, 1951a), hemolymph coagulation in a number of insects appears to be a continuous process, initiated by alterations taking place rapidly in a category of highly labile hemocytes (coagulocytes). These alterations are followed by the development of islands of coagulation in the surrounding plasma. Various degrees of extension of the process occur in different species of insects, from a general clotting, macroscopically visible, to a limited reaction, consisting of a few microscopic islands of coagulation, centered by these hemocytes and scattered in an apparently fluid hemolymph.

The aim of the present study was to investigate by means of the phase contrast microscope the reactions of the insect hemolymph to substances or physical conditions inhibiting the coagulation process in vertebrates and other invertebrates. Insects, chiefly Orthoptera, characterized by a conspicuous clotting process, were selected as experimental material.

The appearance or the absence of the early alterations taking place in the category of highly labile hemocytes and in the neighboring plasma was found to be an expedient test for the evaluation of the degree of interference with the coagulation process brought about by various compounds and experimental conditions.

## MATERIAL AND METHODS

The species of insects used for the present investigations were *Locusta viridissima* (great green grasshopper), *Chorthippus* sp. (short-horned grasshopper), *Gryllulus domesticus* (house cricket), *Periplaneta americana* L. (cockroach), *Blabera gigantea* (South American cockroach), *Mantis religiosa* (praying mantis), *Gryllotalpa gryllotalpa* (mole-cricket), *Carausius morosus* (stick insect) (Orthop-

---

[1] Abstracts of preliminary results were delivered at the International Anatomical Congress, Oxford, July 24–28, 1950, and at the XIXth meeting of the Association des Physiologistes de Langue française, Liège, October 1951.

The author is greatly indebted to Madame Dr. Gh. Duchâteau for controlling the pH of the solutions tested, to Messrs. Alrose Chemical Co., Providence, R. I., to Prof. Z. M. Bacq, to Messrs. Belgopharma and "Bayer-Leverkusen," to Prof. P. Govaerts, to Messrs. Pharmacie Centrale de Belgique, to Prof. R. Pottier (Union Chimique Belge) and to Messrs. Roche, S. A., for kindly supplying various substances.

The expenses of the research were partly defrayed by the Fonds National de la Recherche Scientifique. The charges for three plates were generously supported by Messrs. Wild, Heerbrugg, St. Gall, Switzerland.

tera); *Forficula auricularia* (earwig) (Dermaptera); *Nepa cinerea* (water-scorpion), *Lethocerus cordofanus* Mayr (Belostomatidae, Belgian Congo) (Hemiptera); *Meloe proscarabaeus* (oil beetle), *Tithoes frontalis* (Longicorn from Belgian Congo) (Coleoptera); and *Diprion pini* (Hymenoptera).

Thirty-three substances were tested. Most of them are anticoagulants on vertebrate blood *in vivo* or *in vitro*. These substances were arranged in the summarized report of the data below according to MacIntosh's (1949) classification of their mode of action on vertebrate blood. In addition, the disodium salt of ethylene diamine tetraacetic acid (Sequestrene NA 2: Alrose Chem. Co.: Dyckerhoff, Marx and Ludwig, 1942; Proescher, 1951), Apikur (Lenggenhager, 1949), hexamethylene-glycol (Foster, Samsa, Shulman and Ferry, 1951; Shulman and Ferry, 1951), the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside or Treburon, Ro2–3053 (Mangieri, Engelberg and Randall, 1951), recently reported as anticoagulants of vertebrate blood, were also studied. We were unable to obtain hirudin and novirudin.

For study the reagents were dissolved or diluted at various concentrations with Meisenheimer's fluid, a special Ringer solution for insects (Bi-distilled water: 1000 gm.; NaCl: 7.5 gm.; $NaHCO_3$: 0.1 gm.; KCl: 0.2 gm.; $CaCl_2$: 0.2 gm.) This fluid was found to be a relatively good preservative of the cellular structures and did not interfere with the reactions. Distilled water was also used. The pH of the solutions was controlled before use.

Droplets of hemolymph issuing from the severed end of an antenna or a leg were allowed to fall in approximately the same volume of solution placed in the middle of a standard microscopical slide (Gold Seal glass). Preparations were also made by dipping the severed antenna directly into the solution. The mixture of hemolymph and solution, approximately 0.005 to 0.01 cc. in total volume, was homogenized by gentle lateral shakings of the slide. A thin Gold Seal coverglass, wet in its middle part with a droplet of the solution, was thus gently placed down onto the slide, droplet against drop of mixture. Spontaneous spreading gave a film of suitable thinness for observation. With this procedure, the labile hemocytes were generally protected from contact with glass surfaces before being thoroughly mixed with the solution of the inhibitor.

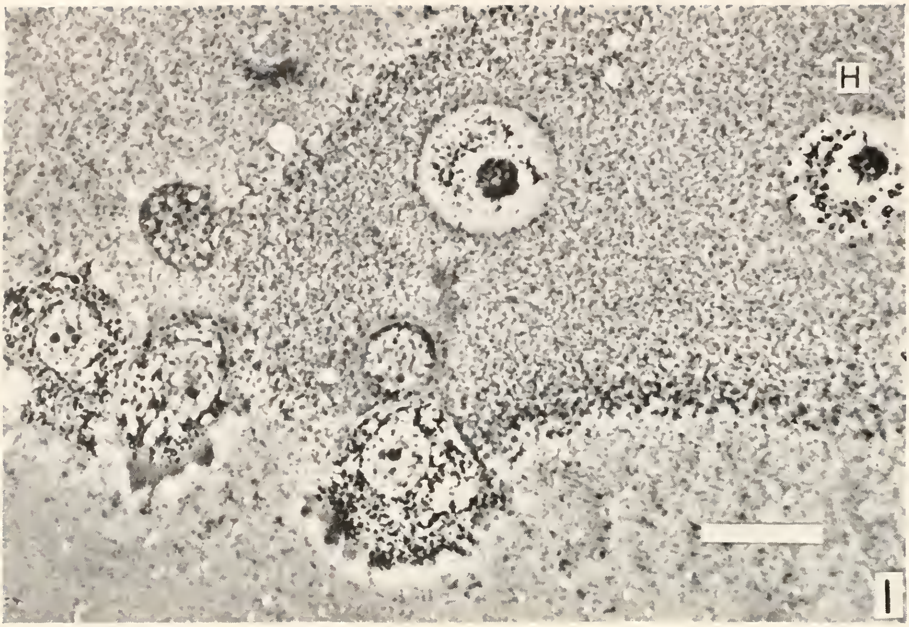
Control preparations were made with Meisenheimer's fluid alone at various pH values in order to appreciate the part played by the dilution of the hemolymph and by the pH in the modifications of the coagulation pictures.

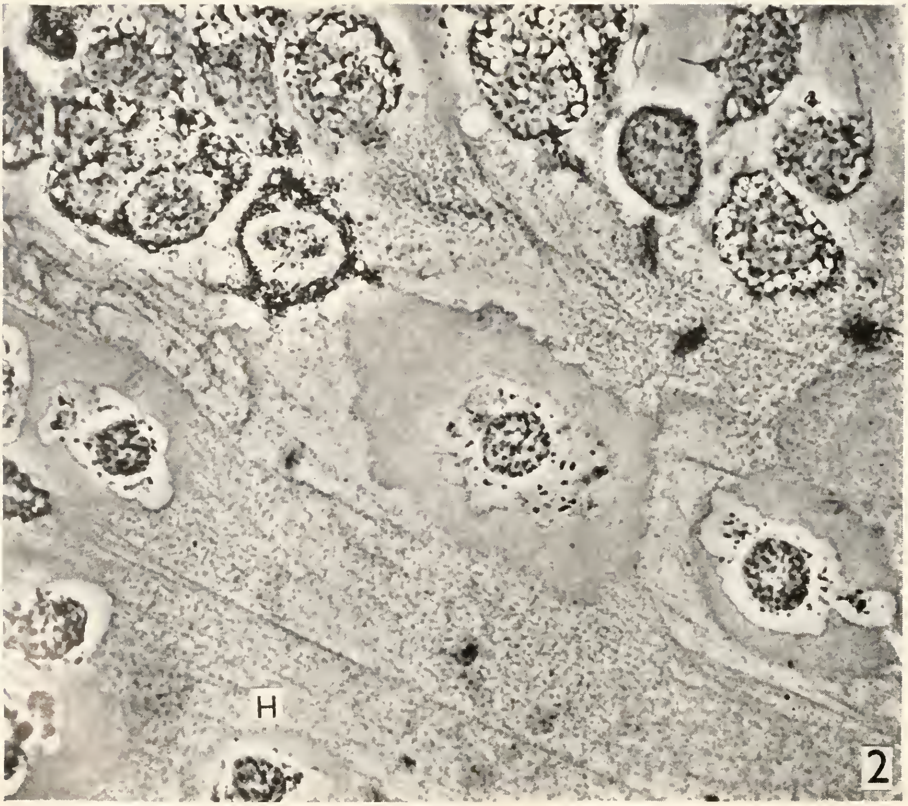
Reactions of the hemolymph to contact with surfaces of various plastics (cellophane, acetophane, plexiglass, Lucite) or with glass made hydrophobic by coating with paraffin, formvar, parlodion and silicone G. E. Drifilm No. 9987, were also studied.

Silicone coatings of glass were prepared according to the standard technique used with vertebrate blood, Jaques *et al.* (1946). The preparations, stored in moist chambers, were observed during several hours.

## RESULTS

Nine hundred tests approximately were performed with the substances listed in the summarized report of the data (see below).


H
1


H
2

*Control of the effects of dilution*

The degree of dilution of the hemolymph with the Meisenheimer's Ringer used in the present experiments (50%) did not interfere with the process of coagulation. The successive stages previously described in the normal hemolymph were also observed to take place after dilution. The highly labile hemocytes were characterized by a relatively small, sharply outlined nucleus, a pale hyaline cytoplasm, in which a few black granular particles were scattered. Within a few seconds, these hyaline hemocytes underwent a succession of intense modifications in their cytological structure: in the mole-cricket, for instance, the changes consisted of a rapid expansion of the cytoplasmic substance, with occasional beam-like bulging of cytoplasmic blisters and blebs (see Grégoire, 1951a; Figs. 2, 3, 6, 7, 8, 12). These alterations were accompanied or followed by the appearance in the surrounding plasma of a thin fog, developing into a circular cloud made of granular particles and progressively increasing in amount and in density. Variable extension of the process and organization of the coagulum into a delicate meshwork of fibrils took place subsequently (Figs. 1 and 2).

The difference between undiluted and diluted materials consisted chiefly of a decrease, proportional to the degree of the dilution, in the size of the islands and in the density of the coagulating materials (see Grégoire, 1951a; Figs. 1 and 2).

By spreading out between slide and coverslip, the diluted coagulum often assumed the shape of thin granular and elastic veil-like structures, a type of picture described elsewhere (Grégoire, 1951a; Figs. 24 and 26) in insect species belonging to various orders of insects (Coleoptera, Lepidoptera). The laying of the coverglass onto the slide often brought about a drawing of the coagulum into strings or threadlike fibrils. In the species used in the present studies, veils and strings were subsidiary morphological aspects induced by mechanical agencies.

---

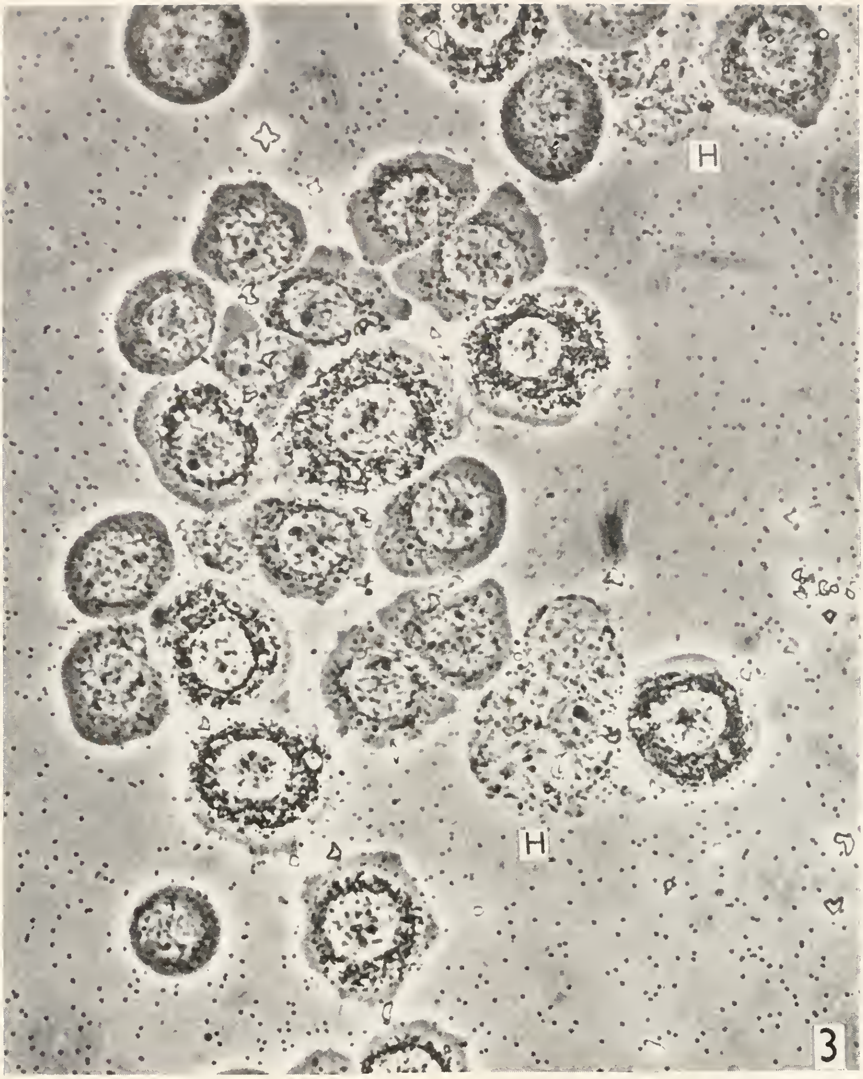
In most preparations, unless otherwise specified, one drop of hemolymph is added to one drop of a solution in Meisenheimer's Ringer of the substance to be tested, lying on a microscopical slide. The mixture, homogenized by lateral shaking of the slide, is allowed to spread out as a thin film between slide and coverglass.

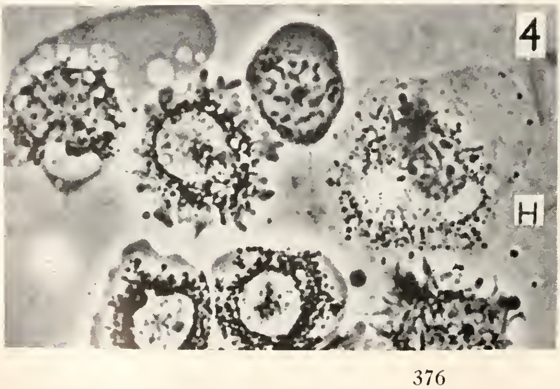
All the microphotographs were taken by phase contrast microscopy (P. C. M. *Wild* M/10, Heerbrugg, Switzerland, with the objectives Ph 7/0.20 and Ph 40/0.66; Microcamera Leitz/Makam) and subsequently enlarged. The scale on the pictures represents 20 microns.

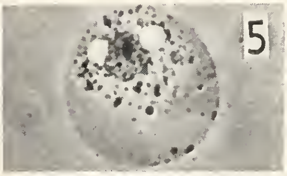
Variations in size of the hemocytes, as observed in the present pictures, are unreliable: they depend chiefly on adhesiveness factors varying with the amount of hemolymph, the area of spreading out, the thickness of the film and the local pressure of the coverglass onto the slide.

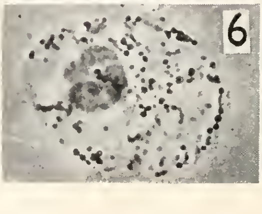
FIGURE 1. Mole-cricket (*Gryllotalpa gryllotalpa*, Orthopt.) Undiluted hemolymph, 20 minutes after withdrawal. Two hyaline hemocytes (H) are seen surrounded by a coagulation island. Extension of the process of coagulation at the upper left and lower right of the picture. Three granular hemocytes and two small round cells are being passively embedded in the extending granular coagulum. In the control preparations of the dilution factor, identical reactions can be observed, but some of the coagulation islands are of smaller size than in undiluted preparations (compare with Grégoire, 1951a, Figs. 1 and 2). × 800.

FIGURE 2. American cockroach (*Periplaneta americana* L., Orthopt.). Undiluted hemolymph, 12 minutes after withdrawal. Dense coagulation islands around several hyaline hemocytes. In the lower half of the picture, area of extension and of organization of the coagulum into a granular meshwork. About 13 elements, belonging to the other categories of hemocytes (granular, small round cells and transition forms) are passively embedded in the coagulum. × 800.


H
H
3


4
H


5


6

### *Control of the factor pH of the solution investigated*

Dilution of the hemolymph with Meisenheimer's fluid, brought to different pH covering the ranges of pH recorded in most solutions of the substances investigated, did not interfere with the process of coagulation. However, when hemolymph was diluted with Meisenheimer's fluid at pH 0.6 and 1.20, precipitates and a decrease in the amount of the coagulating materials were occasionally recorded. On the other hand, dilution with Meisenheimer's fluid at pH 9.45 appeared to induce an increase in the amount of the coagulum. This effect of alkalinity has already been pointed out (Muttkowski, 1924; Beard, 1950).

### *Microscopic aspects of the coagulation inhibition*

The alterations taking place in the presence of potassium oxalate (at the concentration of 0.2% up) may be selected for the description of the total inhibition of the process of coagulation brought about by various compounds.

When the hemolymph was mixed with a solution of this substance, the hyaline hemocytes failed to undergo the alterations observed with the normal or the diluted blood. They appeared as pale spherical or oval cucumber-like bodies, loosely fixed to the glass or floating freely (Figs. 3 to 6, 15, 20 and 21).

Occasional rupture of these hyaline hemocytes, discharge of their cytoplasmic substance or granules did not bring about any change in their surrounding plasma; neither did contact of these elements (Figs. 18, 19) with foreign bodies, which in the normal blood rapidly induced the local formation of a coagulation island.

The other categories of hemocytes (small round or so-called stem cells, various kinds of granular hemocytes and transition forms), scattered or agglutinated in clusters at random, appeared also as spherical, oval or polygonal elements (Fig. 3). They occasionally spread out moderately and sent short spiky pseudopodia or bulging blebs (Fig. 4).

The oxalate type of reaction was observed in mixtures of equal amounts of hemolymph and solutions of potassium oxalate (0.2% up; Figs. 3 to 6, 11, 15, 20 and 21), sodium citrate (1%; Fig. 7), saturated magnesium sulfate, Sequestrene NA 2 (0.1% up; Figs. 12, 22, 23); of organic esters of sulfuric acid: Treburon[2] (1% up), Suramin (Belganyl: 0.1% up; Fig. 13); Bayer 205 (Germanine:

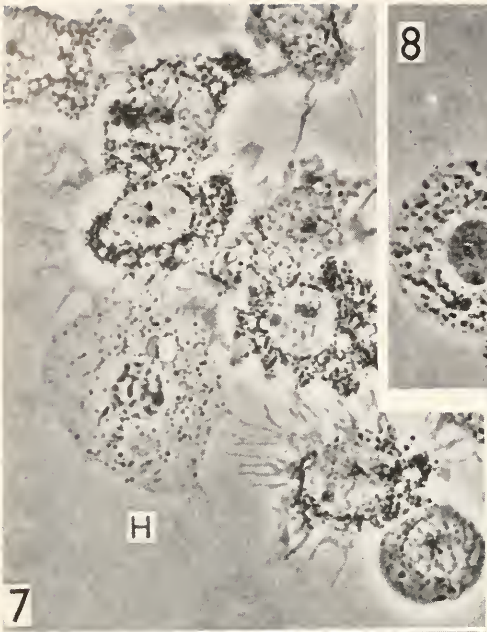
[2] A 0.1% solution of benzophenol, corresponding to the amount of antiseptic contained in the Treburon and heparin vials used, did not interfere with the coagulation process, in control preparations.

---

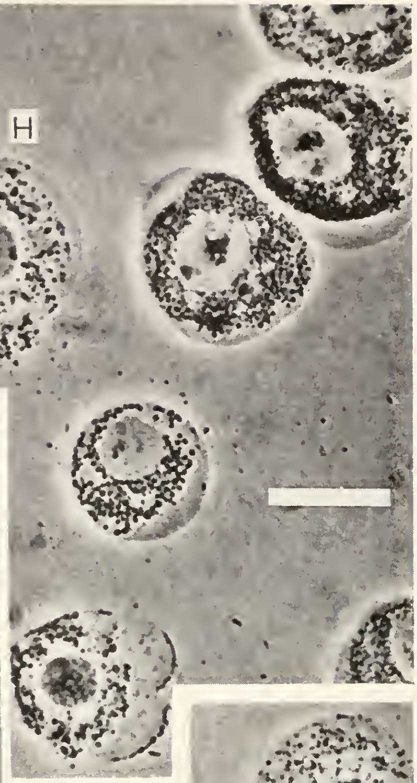
FIGURE 3. *Gryllotalpa.* Twenty-nine minutes after addition of three drops of hemolymph to one drop of a 1% solution of potassium oxalate (pH 7.60). No plasma reaction around two hyaline hemocytes. The other categories of cells are loosely agglutinated. Some send out thin spiky pseudopodia. The black dots in the plasma are precipitates of calcium oxalate. × 800.

FIGURE 4. *Gryllotalpa.* Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 80 minutes after mixture. No plasma reaction around a spread hyaline hemocyte. Two granular hemocytes with bulging blisters. × 800.

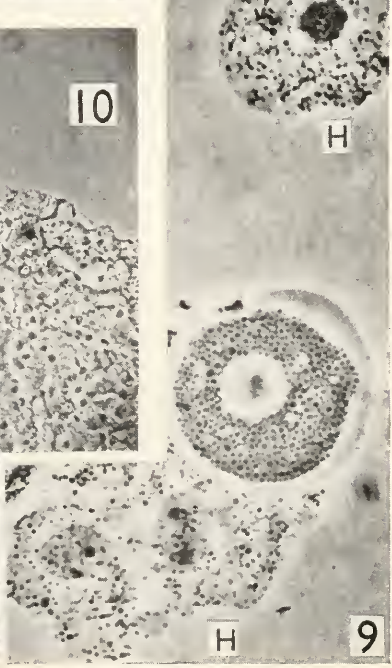
FIGURES 5 AND 6. *Gryllotalpa.* Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 28 and 60 minutes after mixture. No plasma reaction around two swollen hyaline hemocytes. Owing to the active motion of the intra-cytoplasmic granules, these structures lack sharpness in the picture. × 800.



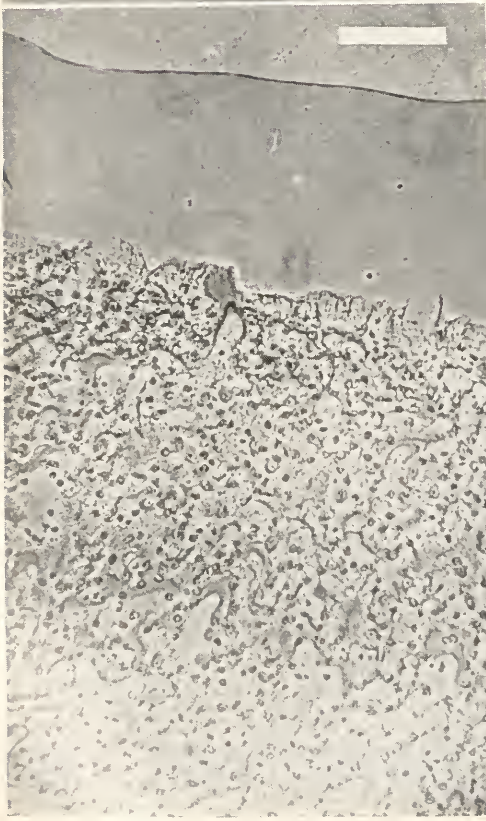
7



8



9



10

0.2% up), Chicago Blue 6 B (0.1% up, with *Periplaneta* hemolymph; 1% up, with *Gryllotalpa* hemolymph; Fig. 14), Liquoid (1% up; Fig. 19), Chlorazol fast pink BKS (0.02% up); of organic bases and basic dyes: Methylene blue (2%), Janus green B (0.02% with *Periplaneta* hemolymph, 0.2% with *Gryllotalpa* hemolymph); of reducing substances: sodium bisulfite (2% up), sodium thiosulfate (2% up; Figs. 17 and 18), sodium hydrosulfite (3%). With 0.1% solutions of potassium oxalate and concentrations lower than 1% of sodium citrate (0.3%, 0.4%), definite inhibitory effects were recorded, but they were not constant.

Absence of coagulation was recorded with a 10% solution of Lanthanum chloride, with solutions of cocaine hydrochloride (1% up; Figs. 8 and 9) and with a 10% solution of DDT.

In mixtures of hemolymph with solutions of Heparin,[2] diethylamine, methyl violet, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, sodium taurocholate, Apikur, hexamethylene-glycol, a great deal of variation was recorded in the reactions, and anticoagulant effects of the oxalate type could not be consistently observed.

In several tests, there was a definite lack of coagulation, especially when using heparin or solutions at high concentration of acidified diethylamine (10%) alkalinized glutathione (10%), alkalinized cysteine hydrochloride (10%), peptone (10% up) and sodium taurocholate (16% up).

On the other hand, a 10% solution of cysteine hydrochloride (pH 0.60) seemed to increase the rapidity of initiation of the process of coagulation and the amount of coagulum.

In other tests performed with the same substances, the process was not totally prevented, as in the oxalate type, but was definitely reduced; thin coatings of coagulating substance developed around a limited number of hyaline hemocytes; the clotted materials were decreased in amounts when compared with the corresponding formations in the dilution controls. Extension of the coagulation process from around the coagulation islands did not occur or was limited.

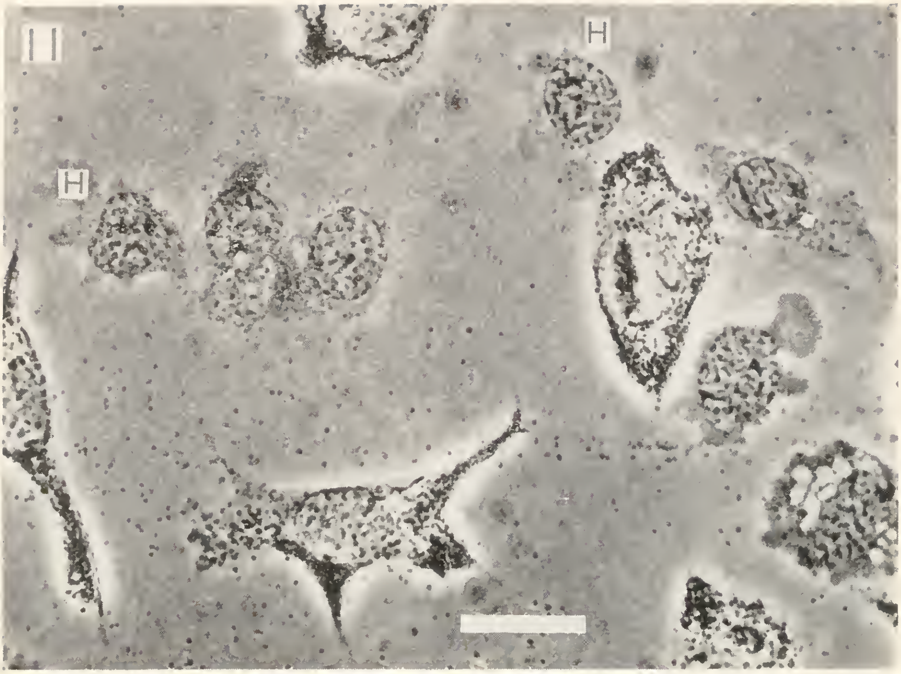
At the concentrations used in the present work, various solutions of saccharose, tricalcium phosphate, protamine, nucleic acid, sodium nucleinate did not interfere with the process of coagulation. However, pictures of inhibition were occasionally observed with solutions of sodium nucleinate (Fig. 16).

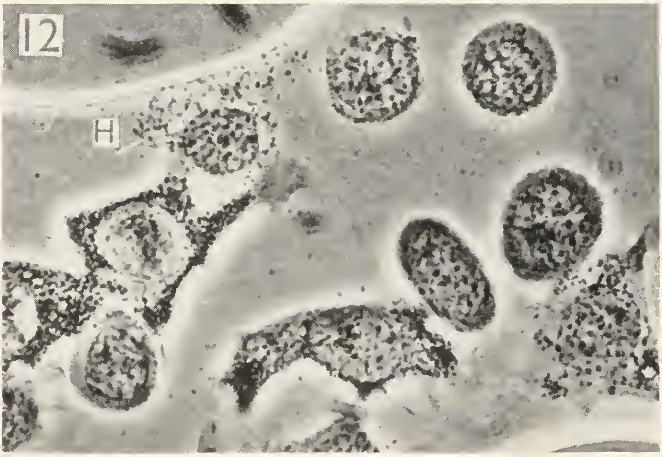
---

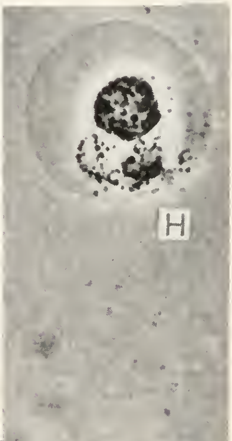
FIGURE 7. *Gryllotalpa.* Hemolymph added to a 1% solution of sodium citrate (pH 7.58) two hours after mixture. No plasma reaction around the hyaline hemocyte, containing intracytoplasmic granules in intense motion. Spreading out of the hyaloplasm of the granular hemocytes. × 800.

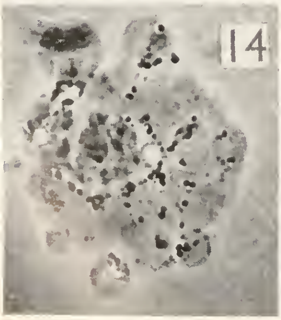
FIGURES 8 AND 9. *Gryllotalpa.* Ten and five minutes after addition of hemolymph to a 1% solution of cocaine hydrochloride (pH 6.67 and 6.60). All elements appear swollen and contain granules in active motion. No plasma reaction in spite of occasional (Fig. 8, H) discharge by the hyaline hemocyte of cytoplasmic substance and granules. × 800.

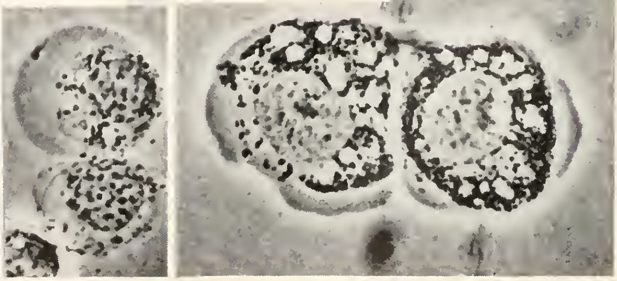
FIGURE 10. *Gryllotalpa.* Hemolymph clot between slide and coverglass coated with three sheets of Silicone G. E. Drifilm No. 9987, 105 minutes after withdrawal from antenna. A part of the contracting discoidal gel (see text) surrounded by a ring of fluid serum. At the borderline of the clot, the last retracting fibrils are seen. Within the clot, numerous little grey patches are coagulation islands centered by hyaline hemocytes. The scale represents 200 microns. × 70.


11


12


13


14


15

### *Special reactions of the hemolymph components to the different substances tested*

Differences were observed in the degree of preservation of the cytological structures of the hemocytes, when the hemolymph was mixed with solutions of the substances investigated. A satisfactory preservation of the cell integrity was obtained with solutions of potassium oxalate, sodium citrate, Sequestrene and cocaine hydrochloride. Various degrees of damage to the cytological structures were occasionally recorded with heparin, Liquoid,, Chicago Blue (2 to 15%). Solutions of sodium taurocholate (2–15%), hexamethylene-glycol (10–15%) and diethylamine (2% up) brought about dissolution of the formed elements. Milky precipitates were observed to develop frequently when the hemolymph was mixed with solutions of Liquoid, Janus green, methyl-violet and yeast nucleic acid.

In tests performed with diethylamine and protamine, the plasma reaction was characterized by the constitution of meshworks of thin elastic fibrils on which granular (precipitate) materials were absorbed.

### *Reaction of the hemolymph to hydrophobic surfaces*

No modification nor delay in the process of coagulation was observed when the hemolymph was collected on non-adhesive surfaces of various resins and plastics (cellophane, acetophane, plexiglass, Lucite) or on glass coated with olive oil, paraffin, parlodion, formvar or with one and several sheets of silicone. Neither could coagulation be prevented when an oiled antenna was severed with oiled scissors or when the whole procedure of removing the blood was performed under oil.

When a coated coverslip was applied to the semi-spherical drop of hemolymph lying on a non-wetable slide, there was only a limited circular spreading out of the drop under the weight of the coverglass. Coagulation took place rapidly and the clot appeared as a thick elastic discoidal gel with sharp boundaries. This gel retracted progressively and within a few minutes it appeared surrounded by a ring of exuded serum (Fig. 10).

## DISCUSSION

### *Reliability of the procedure used*

In the present study, the development or the absence of alterations in a category of hyaline hemocytes and in the plasma directly surrounding these elements was

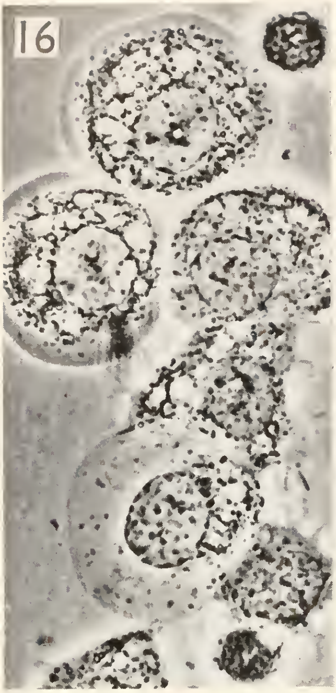
FIGURE 11. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 0.5% solution of potassium oxalate (pH 7.60). No plasma reaction around several hyaline hemocytes with unsharp cytoplasmic outlines or disintegrated to naked nuclei surrounded by fragments of cytoplasm. Elements belonging to other categories of hemocytes have undergone various modifications (lappet-like pseudopodia, vacuoles, cytoplasmic buddings). × 800.

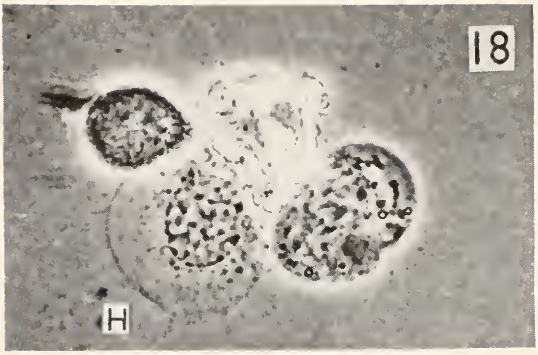
FIGURE 12. *Periplaneta.* Twenty minutes after addition of hemolymph to a 0.1% solution of Sequestrene NA 2 (diluted from a 2% solution at pH 4.10). No plasma reaction around the hyaline hemocytes. × 800.

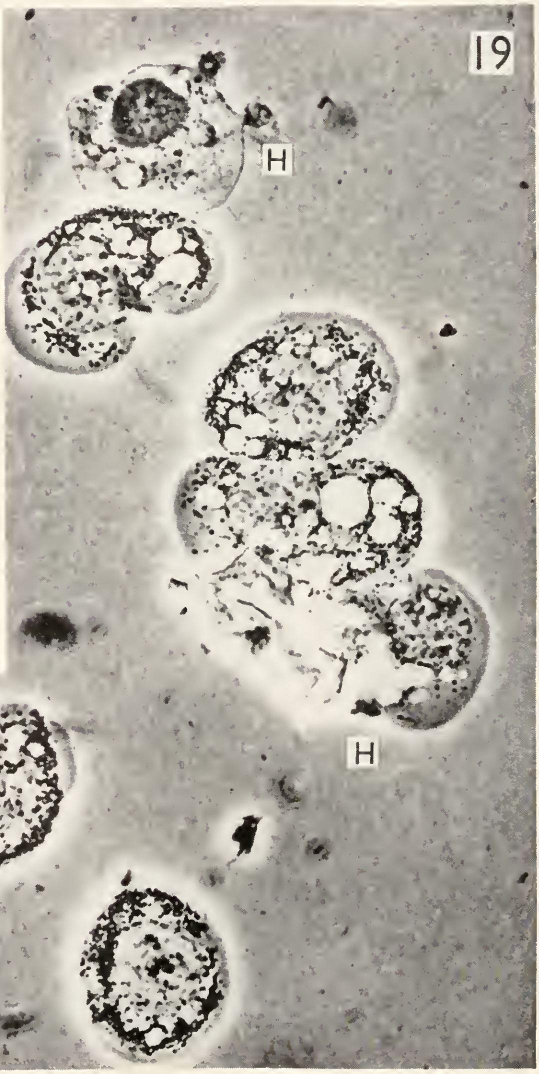
FIGURE 13. *Periplaneta.* Forty minutes after addition of hemolymph to a 0.1% solution of Belganyl-Suramin, diluted from a 2% solution at pH 7.37. A hyaline hemocyte and two granular hemocytes. No plasma reaction. × 800.

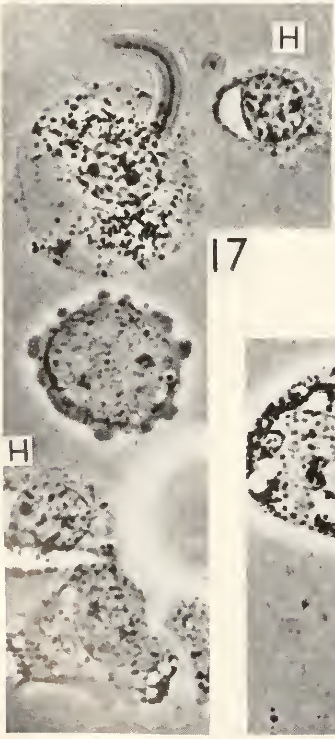
FIGURE 14. *Gryllotalpa.* Sixty-five minutes after addition of hemolymph to a 1% solution of Chicago Blue 6 B (pH 8.66). No plasma reaction around an altered hyaline hemocyte (peripheral cytoplasmic blisters). × 800.

FIGURE 15. Praying mantis (*Mantis religiosa,* Orthopt.). Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate. No plasma reaction around two hyaline hemocytes. In control preparations (see Grégoire, 1951a, Figs. 9, 10 and 11) all elements of this category are surrounded rapidly by dense coagulation islands. × 800.


16


18
H


19
H
H


H
17
H

the test selected for appreciating the anticoagulant effects of different substances on insect hemolymph.

In spite of this rather simple criterion the following pitfalls could conceivably interfere with a correct appreciation of the results:

1) The extreme speed of coagulation of shed hemolymph which characterizes different insects constitutes a serious handicap in any experimental study of the process in these animals (Beard, 1950). This property of the insect blood is related to the high degree of lability exhibited by the hyaline hemocytes (coagulocytes) to contact with foreign environment (Grégoire and Florkin, 1950; Grégoire, 1951a): in these conditions, coagulation can easily take place at the wound site before hemolymph reaches the solution of inhibitor or is thoroughly mixed with it (Beard, 1950). In the latter case, microscopic areas of normal coagulation can develop at the site of falling of the drop into the anticoagulant solution, especially when hyaline hemocytes, still bathed in their normal plasma environment, accidentally meet tiny foreign bodies, such as dust particles or pieces of chitinous debris. Dipping antennas or legs into the solution before severance, immediate shaking to insure dispersion of the formed elements and thorough contact between the labile hemocytes and the inhibitor were found to decrease the occurrence of these accidents. This procedure, however, prevents accurate evaluation of the amount of blood flowing into the solution.

2) Stirring of the mixtures by shaking before the coverglass is applied does not completely prevent local variations in the degree of plasma dilution in different areas of the film spread out between slide and coverslip. Absence of islands of coagulation around hyaline hemocytes in the areas of higher dilution, usually in the peripheral parts of the preparations, can give deceptive pictures of inhibition. Attempts to appreciate this factor were performed in mock preparations, in which solutions of colloidal dyes (*e.g.*, india ink) were used instead of hemolymph and submitted to the same mechanical agencies. The distribution under the coverglass of the areas with various densities of the diluted dye was recorded, the stirring repeated on similar tests until films of uniform tone were obtained, and the same mechanical procedure applied to the diluted hemolymph.

3) The present results have been established on standard preparations consisting mostly of approximately equal amounts of hemolymph and solutions to be tested. It is possible that decreasing the relative amount of hemolymph or increasing that of the solutions would allow one to observe anticoagulant effects for substances for which none has been recorded in the present conditions.

---

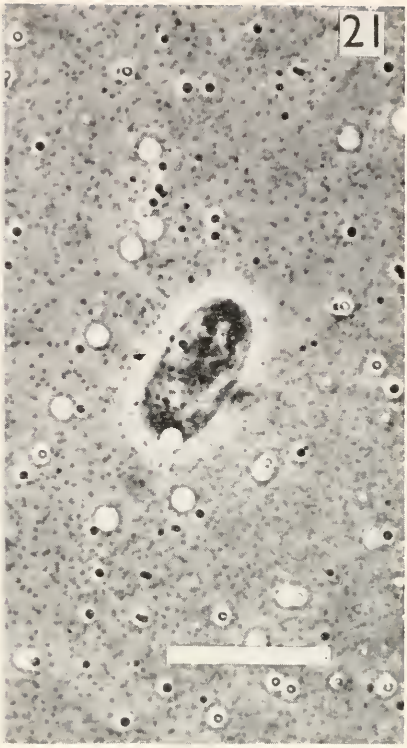
FIGURE 16. *Periplaneta.* One hundred and ten minutes after addition of hemolymph to a 2% solution of sodium nucleinate Merck (pH 5.50). Area without plasma coagulation (exceptional). Seven hemocytes including a swollen hyaline hemocyte. × 800.

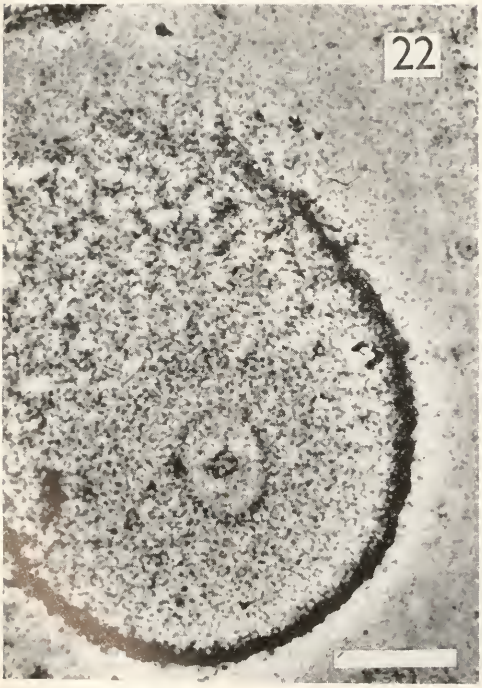
FIGURE 17. *Periplaneta.* Sixty minutes after addition of hemolymph to a 2% solution of sodium thiosulfate in distilled water (pH 6.25). No plasma reaction around hyaline hemocytes. The large hyaline cell with moving intra-cytoplasmic granules belongs probably to the same category. × 800.

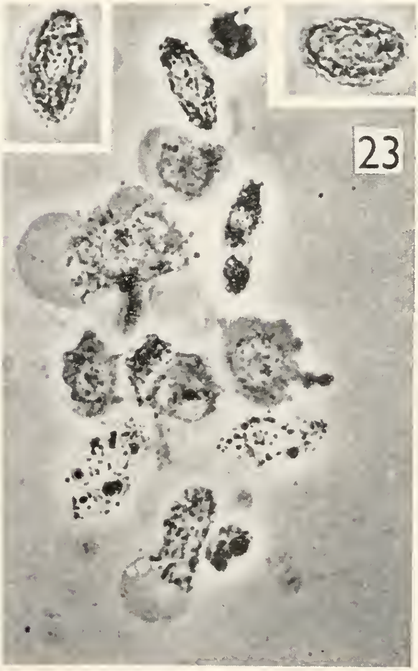
FIGURE 18. *Periplaneta.* Preparation as in Figure 17, after 70 minutes. There is no plasma reaction around the hyaline hemocyte anchored to a foreign body (piece of chitinous debris). In normal hemolymph, contact with a foreign body would rapidly induce production of a coagulation island around such a hyaline hemocyte. × 800.

FIGURE 19. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 1% solution of Liquoid (pH 7.60). No plasma reaction around two hyaline hemocytes, one of them attached to a foreign body. × 800.

20


21


22


23

In addition, the threshold of efficiency of various substances seems to vary with the species of insect used: for instance, as seen in different tests, for preventing coagulation in *Gryllotalpa* hemolymph, it was often necessary to use higher concentrations of anticoagulant solutions than for obtaining inhibition of the coagulation of *Periplaneta* hemolymph, with the same substance.

### *Comparative study of the literature*

Coagulation of insect hemolymph has been reported to be prevented by various organic acids: nucleic (Paillot, 1923), citric and ascorbic acids (Beard, 1950), vapors of acetic and several fatty acids (Shull, Riley and Richardson, 1932; Fischer, 1935; Shull, 1936); also by surface-active and physical agents, such as heating of the animal body (Fredericq, 1881; Yeager, Shull and Farrar, 1932; Babers, 1938; Beard, 1950), freezing and ultrasonic waves (Beard, 1950).

On the other hand, failure of several anticoagulants of mammalian blood to inhibit the coagulation of the insect hemolymph has been reported for magnesium sulfate, (Fredericq, 1881), potassium oxalate (Muttkowski, 1924; Yeager, Shull and Farrar, 1932), heparin, hirudine, citrates, dicoumarol (*in vivo:* Beard, 1950). Hemolymph coagulation was not affected by cyanide fumes (Muttkowski, 1924; Shull, Riley and Richardson, 1932), nor by calcium cyanide (Beard, 1950).

The present results are at variance with the former data obtained with potassium oxalate, citrate and to some extent with heparin.

The discrepancies could be explained by differences in the experimental conditions (for instance, concentrations and relative amounts of the inhibitors used). As the literature on the process of normal coagulation in insects is controversial, it is more probable that the standards used for determining an anticoagulant effect cannot be compared.

The normal hemolymph coagulation of different insects has been described as being either a cellular agglutination or a plasma coagulation, both considered as two physiologically distinct processes, which can occur independently or together in the same insect or in different insects (see Beard, 1950).

In a recent paper, Beard (1950) refers to the difficulties encountered in appreciating the effects of the coagulation inhibitors on insect blood, while with the clotting of mammalian blood, fibrin formation is a convenient end-point for judging

FIGURE 20. Water scorpion (*Nepa cinerea,* Hemipt.). Undiluted hemolymph, 25 minutes after withdrawal. A large circular island of coagulation is seen surrounding an altered hyaline hemocyte. × 1080.

FIGURE 21. *Nepa.* Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate (pH 8.10). A cucumber-like inert hyaline hemocyte is seen floating freely in the fluid plasma. Numerous grey and black dots are moving precipitates of calcium oxalate. × 1080.

FIGURE 22. *Lethocerus cordofanus* Mayr (Belostomatidae from the Belgian Congo). Undiluted hemolymph, 25 minutes after withdrawal. A hyaline hemocyte is seen in the center of a circular island of coagulation. × 800.

FIGURE 23. *Lethocerus.* Eighty minutes after addition of hemolymph to a 0.5% solution of Sequestrene NA 2. No plasma reaction. A group of variously altered elements belonging to different categories of hemocytes. Owing to the shrinkage, hyaline hemocytes are difficult to recognize unquestionably: two pale cells (lower part of the picture) and two elements isolated in squares are probably hyaline hemocytes. × 800.

the effect. According to Beard, dispersion of the formed elements is the desired response in searching for an inhibitory agent.

As it appears from our previous and present studies, a process of cellular agglutination does not play any part in the phenomenon of hemolymph coagulation in the species of insects used in the present experiments. The categories of blood cells other than the hyaline hemocytes are inert, scattered or agglutinated at random and passively embedded in the plasma coagulum. Accordingly, the dispersion of the formed elements could not be selected as a test for measuring an inhibiting effect on the process of hemolymph coagulation in insects.

The present results, on the other hand, are in agreement with various data obtained with crustacean blood, as regards the inhibiting effect on the true plasma coagulation (so called second coagulation) of magnesium sulfate (Halliburton, 1885; Ducceschi, 1903, 1915), potassium oxalate (Bottazzi, 1902; Ducceschi, 1903, 1915; Loeb, 1903, 1904; Nolf, 1909; Parsons and Parsons, 1923), highly concentrated solutions of peptone (Bottazzi, 1902; Loeb, 1903; Nolf, 1909; Parsons and Parsons, 1923), sodium hydrosulfite (Hensill, 1948) and cocaine hydrochloride (Ducceschi, 1903, 1915).

### *Mechanism of the coagulation inhibiting effect in insect hemolymph*

Little is known of the chemical nature of the substances involved in the process of hemolymph coagulation in insects. The terms fibrin and gelatine used in the literature for characterizing different aspects of the insect clots rather represent morphological analogies with the corresponding compounds in crustacean or vertebrate blood.

An anticoagulant can act on insect hemolymph either on the hyaline hemocytes involved in the initiation of the process or on the coagulable compounds contained in the plasma, or on both factors.

From the present data, different anticoagulants can be efficient in preserving the extremely labile hyaline hemocytes from the alterations which they rapidly undergo in normal hemolymph when they come in contact with glass surfaces and foreign particles and which distinguish this category of blood elements from the other less labile hemocytes. In mixtures of hemolymph and efficient inhibitors, the hyaline hemocytes generally keep a spherical or oval shape and do not spread out. Inconstant anticoagulants are unable to prevent completely the alterations of all the hyaline hemocytes and the subsequent reaction of the plasma in their vicinity: the coagulation process is scattered and the areas of extension of the coagulum are decreased by various degrees.

As often suggested for a corresponding category of elements in crustacean blood (Halliburton, 1885; Hardy, 1892; Ducceschi, 1903; Tait and Gunn, 1918) the hyaline hemocytes of insects could take part in the coagulation process by yielding coagulation—inducing substances. Anticoagulants would prevent the release of these active substances. However, in incidental observations, no clotting reaction could be detected in the plasma around some hyaline hemocytes which had discharged a part of their cytoplasmic substance or were reduced to naked nuclei after their rupture. This result could be explained as well by inactivation of the coagulation-inducing substances contained in these hemocytes as by alterations of one or more plasma components involved in the coagulation process.

Of six compounds which act on vertebrate blood as removers of calcium ions, deionizing agents or preventers of platelet disintegration, five were efficient anticoagulants of insect hemolymph. The present results do not allow one to conclude whether these compounds interfere with the insect coagulation process either like other strong salts, in preventing the release of active substances by the hyaline hemocytes, or in inactivating these substances, or by removing calcium salts possibly involved in the mechanism of the clotting process.

In preliminary experiments, a calcium chloride solution was allowed to flow into a film of oxalated hemolymph. When the calcium chloride reached the spherical, inert hyaline hemocytes bathed in the oxalate solution, these elements underwent sudden modifications, consisting of shrinkage or the extension of adhesive pseudopodia. A definite reaction was not observed in the plasma directly surrounding these elements. Owing to the formation of precipitates between potassium oxalate and calcium chloride, interfering with the observations, the development of veil-like elastic structures in different areas of the preparations, suggesting a possible clotting reaction, remains questionable and requires further study.

Among the organic esters of sulfuric acid, which were found to be efficient anticoagulants of insect blood in the present study, it is worth mentioning that the trypanocidal drug suramin would be an inhibitor of enzymatic activity, according to a recent finding (Willis and Wormall, 1950).

Several other anticoagulants of vertebrate blood, listed above, were not inhibitors at the concentrations used or brought about various degrees of interference with the process of coagulation.

Owing to the lack of data on the number and on the chemical nature of the coagulation factors in insects, interpretation of the failure of these anticoagulants of vertebrate blood to inhibit the process in insects cannot be given at the present time.

### *Comparative physiology*

The present results offer additional data with regard to the existence of converging functional characters in zoological groups as distant as arthropods and vertebrates: vertebrate platelets (Ferguson, 1934), Hardy's explosive corpuscles in crustacea (Hardy, 1892; Ducceschi, 1903, 1915; Tait and Gunn, 1918) and hyaline hemocytes in insects (Grégoire and Florkin, 1950; Grégoire, 1951a) exhibit specific morphological changes, which are related to the initiation of the plasma coagulation. Substances decreasing, delaying or preventing the alterations in the platelets (Bizzozero, 1882; Bürker, 1904; Wright, 1941; Feissly and Ludin, 1949), in the crustacean explosive corpuscles (Ducceschi, 1903, 1915; Tait and Gunn, 1918), and in the hyaline hemocytes of insects, as shown in the present studies, decrease, delay or prevent the blood coagulation. However, there are differences between vertebrate platelets and insect hyaline hemocytes; the latter elements do not exhibit, at least *in vitro*, the property of auto-agglutinability characterizing the platelets, and their fragility seems to be much greater. In the present investigations, the hyaline hemocytes were not preserved on hydrophobic surfaces, even when they were protected by oily environment from contamination with the tissues at the wound, before falling onto the silicone-coated surface. The oil procedure was successful in the hands of Tait and Gunn (1918) in preventing on plain glass the alterations in the explosive corpuscles and the plasma coagulation in *Astacus* blood.

Consistent failure of the silicone-coated surfaces to prevent coagulation of insect hemolymph seems to indicate that different factors are probably responsible for the induction of the alterations in insect hyaline hemocytes and vertebrate platelets.

### *Substances tested and reactions of insect hemolymph. Summarized report of the data*

For brevity's sake, the reference marks used here below record the following reactions of the plasma:

+: development of coagulation islands around hyaline hemocytes (coagulocytes), followed by various degrees of extension of the process of coagulation;

−: no coagulation, all hyaline hemocytes inert, loosely fixed to the glass or floating in the fluid plasma;

*: development of coagulation islands or veils in limited areas, obviously accidental, induced in the vicinity of foreign bodies, or at the site of falling down of the drop of hemolymph into the solution of anticoagulant (see text);

$: heterogeneous reaction. Areas without coagulation (oxalate type), areas with various degrees in the reduction of the process, as compared with the control preparation in Meisenheimer's fluid. Plasma reaction consisting of tiny coagulation islands or fringes around the hyaline hemocytes, and of thin veils without or with poor extension.

The concentrations of the solutions used are given in %. Unless otherwise indicated, the pH values are those of solutions in Meisenheimer's fluid. The preparations consisted of approximately equal volumes of solution investigated and of hemolymph. The total volume of mixture in each test amounted approximately 0.005 to 0.01 cc. The mixtures were spread out into films under 24 × 24 mm. and 24 × 30 mm. coverslips (see methods above).

1. *Controls of the factors dilution and pH of the solutions of the substances investigated.*

*Meisenheimer's fluid* (incidentally distilled water).
pH 0.6, 1.20, 3.30, 4.12, 4.32, 5.63, 5.83, 7.28, 7.38, 7.83, 8.00, 9.45: 160 tests, all +.

2. *Salts, removers of calcium ions and deionizing agents.*

*Potassium oxalate.*
0.07%: 3 tests $ and 1 test − *.
0.1%: 2 tests $ and 2 tests − *.
0.2%: 8 tests −.
0.5%, 1% (pH 7.60–8.10), 4%, 10% (pH 7.80–8.00) and 20%: 32 tests −.

*Sodium citrate.*
0.1%: 3 tests $ and 2 tests −.
0.3%: 3 tests $ and 3 tests − (incidentally *).
0.4%: 2 tests $ and 6 tests − (incidentally *).
1% (pH 7.58): 14 tests − (incidentally *).

*Magnesium sulfate.*
Saturation (pH 5.86) : 5 tests –.
*Sequestrene NA 2.*
0.1%, 0.5%, 1% and 2% (pH 4.10) : 23 tests – (incidentally *).
*Saccharose* (in Meisenheimer's fluid).
2%, 5%, 10% (pH 7.21) and 50% (pH 7.27) : 7 tests + and 5 tests $.
*Saccharose* (in distilled water).
1% (pH 6.57), 10% (pH 6.40) and 50% (pH 6.20) : 8 tests + and 12 tests $.
*Tricalcium phosphate.*
1% (pH 6.51) : 2 tests +.

3. *Organic esters of sulfuric acid.*

*Heparin* (*Liquemine* Roche).
50 anticoagulant units per drop used (pH 6.50) : 3 tests +, 17 tests $ and 10 tests –.
*Heparin* (powder dissolved in Meisenheimer's fluid).
4800 Toronto anticoagulant units per cc. : 5 tests $ and 3 tests –.
2400 Toronto anticoagulant units per cc. : 2 tests +.
*Treburon* Ro 2–3053/B–3.
1% (*Periplaneta*) : 6 tests – (incidentally *).
2% (*Periplaneta*) : 6 tests – (incidentally *).
1.5% (*Gryllotalpa*) : 1 test $ or – and 1 test –.
3.75%, 7.5% and 15% (*Gryllotalpa* and *Blabera*) : 16 tests – (incidentally *).
*Suramin* (Belganyl).
0.01% : 2 tests $.
0.1%, 0.2% (in distilled water : pH 6.26), 2 % (in Meisenheimer's fluid : pH 7.37) and 10% (in distilled water : pH 6.20) : 12 tests –.
*Bayer 205* (*Germanine*).
0.02% : 2 tests + (*Gryllotalpa*) and 4 tests $ (*Periplaneta*).
0.2% and 2% (pH 7.50) : 12 tests –.
*Chicago Blue* 6 B.
0.1% : 4 tests + (*Gryllotalpa*) and 4 tests – (*Periplaneta*).
1% (pH 8.66) and 10% (pH 8.61) : 18 tests –.
*Liquoid.*
0.01%, 0.02% (pH 7.65), 0.1% (in distilled water : pH 6.43 and in Meisenheimer's fluid : pH 7.82–7.96) : 11 tests +.
0.5% : 2 tests $ and 2 tests –.
1% (pH 7.60) : 6 tests –.
2% (in distilled water : pH 6.40) : precipitates, 3 tests –.
*Chlorazol fast pink BKS.*
0.005% : 2 tests +.
0.01%, 0.02%, 0.05%, 0.2%, 0.5% and 2% (pH 6.00) : 7 tests $ and 21 tests – (precipitates).

4. *Organic bases and basic dyes.*

*Protamine.*
0.1% and 1% (in distilled water: pH 1.83): 8 tests +.
*Diethylamine.*
0.1% (in distilled water: pH 9.29): 6 tests + and 2 tests −.
0.5% (in distilled water: pH 10.61): 6 tests $.
1% (in distilled water: pH 11.07 and in Meisenheimer's fluid: pH 11.83): 2 tests $, 8 tests −. In 2 other tests, dissolution of the hemocytes.
1% (acidified to pH 6.00): 2 tests +, 4 tests $ and 2 tests −.
10% (pH 12.27): 3 tests $ and dissolution of the hemocytes.
10% (acidified to pH 5.80): 8 tests −.
*Methylene Blue.*
0.02% and 0.1% (pH 7.38): 2 tests +, 8 tests $ and 2 tests − *.
1% (pH 3.30): 4 tests $ and 2 tests −.
2% (pH 3.30): 6 tests −.
*Janus Green B.*
0.02%: 2 tests $ (*Gryllotalpa*) and 6 tests − (*Periplaneta*).
0.2% (in distilled water: pH 3.89 and in Meisenheimer's fluid: pH 5.38) and 2% (pH 2.66): 9 tests − and precipitates.
*Methyl violet.*
0.1% and 0.3%: 2 tests +, 1 test $ and 5 tests −.
1% (pH 6.62): 2 tests: precipitates.

5. *Reducing substances.*

*Sodium bisulfite.*
2% (pH 4.49) and 5% (pH 4.30): 8 tests −.
*Sodium thiosulfate.*
2% (pH 6.25) and 10% (pH 5.92): 8 tests − (in 2 tests *).
*Sodium hydrosulfite.*
3% (pH 5.50): 2 tests −.
*Cysteine hydrochloride.*
0.2% and 1%: 3 tests +.
2% (pH 1.10): 5 tests + and 6 tests $.
2% (alkalinized to pH 8.30): 8 tests − (in 4 tests *).
10% (pH 0.60): 5 tests +, 2 tests $ and 2 tests −.
10% (alkalinized to pH 6.92): 8 tests−.
*Glutathione.*
0.2% and 1%: 3 tests + and 1 test $.
2% (pH 2.70–2.85): 3 tests + (*Periplaneta*), 10 tests $ (*Periplaneta*) and 8 tests − (*Gryllotalpa*).
2% (alkalinized to pH 7.30): 8 tests + (*Gryllotalpa*), 8 tests $ (*Periplaneta, Gryllotalpa*) and 8 tests − (*Periplaneta*).
10% (pH 2.61): 2 tests + and 5 tests $.
10% (alkalinized to pH 8.79): 4 tests −.
*l-ascorbic acid cryst.*
2% (pH 2.90): 2 tests $ and 8 tests − *.
10% (pH 2.20): 5 tests $ and 3 tests −.

6. *Salts of rare earth metals.*

*Lanthanum chloride.*
0.2% and 1%: 10 tests + (precipitates).
5%: 2 tests $ and 2 tests − *.
10%: 6 tests − (incidentally *).

7. *Miscellaneous substances.*

*Peptone* (Merck 7213).
2% (in distilled water: pH 7.34 and in Meisenheimer's fluid: pH 7.66): 10 tests $.
5%: 2 tests −.
10% (in distilled water: pH 7.58 and in Meisenheimer's fluid: pH 7.38): 4 tests $ and 9 tests −.
16% (pH 7.60): 5 tests −.

*Sodium taurocholate.*
0.1%, 0.5%, 2% (pH 7.00–7.17), 5% (pH 7.02), and 10%: 3 tests + and 20 tests $.
16% (pH 6.99): 10 tests − (dissolution of the hemocytes).

*Cocain hydrochloride.*
1% (pH 6.17 − 6.67) and 5%: 18 tests − (incidentally *).

*Sodium nucleinate* (Merck).
2% (pH 5.48) and 10% (pH 4.99): 6 tests + and 4 tests $.

*Yeast nucleic acid* (Merck).
0.025%, 0.5%, 1%, 2% (pH 3.00) and 10% (pH 3.48): 13 tests + and in 1 test, precipitates.

*Apikur* (bee venom).
0.015%, 0.03% and 0.07%: 6 tests +, 9 tests $ and 1 test −.
0.15% (pH 3.40): 8 tests $ and 1 test −.

*Hexamethylene glycol.*
2% (in distilled water: pH 5.60 and in Meisenheimer's fluid: pH 6.96), 5% (in distilled water: pH 5.50 and in Meisenheimer's fluid: pH 6.96): 20 tests + and 2 tests $.
10% (pH 6.78): 3 tests $ and 9 tests − (incidentally *).
15% (pH 7.27): dissolution of the hemocytes in all tests.

*D. D. T.*
10%: 8 tests − (incidentally *).

## SUMMARY

1. The effects of 33 substances, most of them anticoagulants of vertebrate blood, have been investigated *in vitro* on the hemolymph of different species of insects, chiefly Orthoptera, exhibiting in normal conditions a conspicuous process of coagulation.

2. The tests (900 approximately) consisted of mixtures in equal amounts of hemolymph and solutions at various concentrations in a special Ringer for insects (Meisenheimer's fluid) of the substances studied. The mixtures were observed by means of a phase contrast microscope after they were spread out in thin films between slide and coverglass.

3. The part played in the reactions by the dilution of the hemolymph and by the pH of the solutions used was appreciated in control preparations made with Meisenheimer's fluid alone at various pH values; no interference with the coagulation process was recorded within the range of pH used (0.60 to 9.45).

4. The morphological criterion selected for appreciating an anticoagulant effect was the development or the absence of alterations in the category of hemocytes especially involved in the initiation of the plasma coagulation, and the development or the absence of coagulation islands around these cells.

5. From the 33 substances tested, 18 were efficient anticoagulants in relatively small amounts; among them were four salts, removers of calcium ions or de-ionizing agents of vertebrate blood, six organic esters of sulfuric acid, including trypanocidal drugs, two basic dyes and three reducing substances. Consistent anticoagulant effects could not be obtained with heparin, and with solutions of the following substances (unless at high concentrations and, for some of them, after acidification or alkalinization): diethylamine, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, Apikur and hexamethylene glycol.

6. Different hydrophobic surfaces, including glass coated with Silicone G. E. Drifilm No. 9987, did not induce any definite modification in the coagulation process.

7. A tentative explanation of the anticoagulant effects is given in the discussion; when the substances tested prevent the morphological alterations in the category of hyaline hemocytes involved in the process of coagulation, this process does not occur. The results bring additional support to the previous interpretation that the hyaline hemocytes play an important part in the initiation of the hemolymph coagulation in different insects.

## LITERATURE CITED

Babers, F. H., 1938. An analysis of the blood of the sixth-instar southern armyworm (*Prodenia eridania*). *J. Agricult. Res.*, **57**: 697–706.

Beard, R. L., 1950. Experimental observations on coagulation of insect hemolymph. *Physiol. Zoöl.*, **23**: 47–57.

Bizzozero, J., 1882. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Arch. f. path. Anat.*, **90**: 261–332.

Bottazzi, F., 1902. Contribution à la connaissance de la coagulation du sang de quelques animaux marins et des moyens pour l'empêcher. *Arch. ital. Biol.*, **37**: 49–63.

Bürker, K., 1904. Blutplättchen und Blutgerinnung. *Arch. f. ges. Physiol.*, **102**: 36–94.

Ducceschi, V., 1903. Untersuchungen über die Blutgerinnung bei wirbellosen Tieren. *Beitr. zur. chem. Physiol. Pathol.*, **3**: 378–384.

Ducceschi, V., 1915. Plaquettes et coagulation du sang. *Arch. ital. Biol.*, **64**: 341–353.

Dyckerhoff, H., R. Marx and B. Ludwig, 1942. Ueber den Wirkungsmechanismus und die Verwendbarkeit einiger blutgerinnungshemmender organischer Substanzen. *Zeitschr. f. ges. exper. Medizin*, **110**: 412–422.

Feissly, R., and H. Ludin, 1949. Action de la cocaine sur les plaquettes sanguines. *Helvet. physiol. pharmacol. Acta*, **7**: (C. R. Soc. Suisse Physiol.) page C 9.

Ferguson, J. H., 1934. Observations on the alterations of blood platelets as a factor in coagulation of the blood. *Amer. J. Physiol.*, **108**: 670–682.

Fischer, R. A., 1935. The effect of acetic acid vapour treatment on blood cell counts in the cockroach *Blatta orientalis* L. *Ann. Entom. Soc. Amer.*, **28**: 146–153.

Foster, J. F., E. G. Samsa, S. Shulman and J. D. Ferry, 1951. The conversion of fibrinogen to fibrin. VI. Flow birefringence studies on clotting systems inhibited by hexamethylene glycol. *Arch. Bioch. and Biophysics*, **34**: 417–436.

FREDERICQ, L., 1881. Sur le sang des insectes. *Bull. Acad. Roy. de Belgique,* 3e série, **1**, no. 4, 487–490.

GRÉGOIRE, CH., 1951a. Blood coagulation in arthropods. II. Phase contrast microscopic observations on hemolymph coagulation in sixty-one species of insects. *Blood,* **6**: 1173–1198.

GRÉGOIRE, CH., 1951b. Sang des insectes et anticoagulants. *J. de Physiologie,* **43**: 888 (Abstract).

GRÉGOIRE, CH., AND M. FLORKIN, 1950. Blood coagulation in arthropods. I. The coagulation of insect blood, as studied with the phase contrast microscope. *Physiol. compar. oecologia,* **2**: 126–138.

HALLIBURTON, W. D., 1885. On the blood of decapod Crustacea. *J. Physiol.,* **6**: 300–335.

HARDY, W. B., 1892. The blood corpuscles of the Crustacea, together with a suggestion as to the origin of the crustacean fibrin-ferment. *J. Physiol.,* **13**: 165–190.

HENSILL, J. S., 1948. Control of coagulation of decapod blood. *Anat. Rec.,* **101**: 736 (Abstract).

JAQUES, L. B., E. FIDLAR, E. T. FELSTED AND A. G. MACDONALD, 1946. Silicone and blood coagulation. *Canad. Med. Assoc. J.,* **55**: 26–31.

LENGGENHAGER, K., 1949. Weitere Fortschritte in der Blutgerinnungslehre. Georg. Thieme Verlag. Stuttgart.

LOEB, L., 1903. On the coagulation of the blood of some arthropods and on the influence of pressure and traction on the protoplasm of the blood cells of arthropods. *Biol. Bull.,* **4**: 301–318.

LOEB, L., 1904. Ueber die Koagulation des Blutes einiger Arthropoden. *Beitr. chem. Physiol. Pathol.,* **5**: 191–207.

MACINTOSH, F. C., 1949. Anticoagulants. *J. Pharm. Pharmacol.,* **1**: 353–367.

MANGIERI, C. N., R. ENGELBERG AND L. O. RANDALL, 1951. The heparin-like activity of a new anticoagulant, Treburon. *J. Pharmacol. Exper. Therap.,* **102**: 156–164.

MUTTKOWSKI, R. A., 1924. Studies on the blood of insects. III. The coagulation and clotting of insect blood. *Bull. Brooklyn Entom. Soc.,* **19**: 128–144.

NOLF, P., 1909. Contribution à l'étude de la coagulation du sang (8e mémoire). La coagulation chez les crustacés. *Arch. Internat. Physiol.,* **7**: 411–461.

PAILLOT, A., 1923. Sur une technique nouvelle permettant l'étude vitale du sang des insectes. *C. R. Soc. Biol.,* **88**: 1046–1048.

PARSONS, T. R., AND W. PARSONS, 1923. Observations on the transport of carbon dioxide in the blood of some marine invertebrates. *J. Gen. Physiol.,* **6**: 153–166.

PROESCHER, F., 1951. Anticoagulant properties of ethylene bisiminodiacetic acid. *Proc. Soc. Exper. Biol. Med.,* **76**: 619–620.

SHULMAN, S., AND J. D. FERRY, 1951. The conversion of fibrinogen to fibrin. *J. Phys. Colloid Chem.,* **55**: 135–150.

SHULL, W. E., 1936. Inhibition of coagulation in the blood of insects by the fatty acid vapor treatment. *Ann. Entom. Soc. Amer.,* **29**: 341–349.

SHULL, W. E., M. K. RILEY AND C. H. RICHARDSON, 1932. Some effects of certain toxic gases on the blood of the cockroach *Periplaneta orientalis* Linn. *J. Econ. Entom.,* **25**: 1070–1072.

TAIT, J., AND J. D. GUNN, 1918. The blood of *Astacus fluviatilis:* a study in crustacean blood, with special reference to coagulation and phagocytosis. *Quart. J. Exper. Physiol.,* **12**: 35–80.

WILLIS, E. D., AND A. WORMALL, 1950. Isoelectric points of enzymes as determined by inhibition with suramin. *Nature,* **165**: 813–814.

WRIGHT, H. P., 1941. The adhesiveness of blood platelets in normal subjects with varying concentrations of anticoagulants. *J. Path. Bact.,* **53**: 255–262.

YEAGER, J. F., W. E. SHULL AND M. D. FARRAR, 1932. On the coagulation of blood from the cockroach, *Periplaneta orientalis* (Linn.), with special reference to blood smears. *Iowa State Coll. J. of Sci.,* **6**: 325–345.

YEAGER, J. F., AND R. W. FAY, 1935. Reducing power of hemolymph from the roach, *Periplaneta americana* Linn., with special reference to coagulation. *Proc. Soc. Exper. Biol. Med.,* **32**: 1037–1038.

# STUDIES ON SHELL FORMATION. II. A MANTLE-SHELL PREPARATION FOR IN VITRO STUDIES[1]

ARTHUR A. HIRATA[2]

*Department of Zoology, Duke University, Durham, N. C., Duke University Marine Laboratory, and U. S. Fishery Laboratory, Beaufort, N. C.*

This report describes a preparation, consisting of an oyster mantle and its associated shell, which has been developed for studying mechanisms of shell formation *in vitro*. The preparation has been found capable of depositing shell of normal structure and can be easily maintained for periods of several days. An account of shell formation by the mantle-shell preparation is presented.

## METHODS

All experiments were carried out with the oyster *Crassostrea virginica* Gmelin.[3] The mantle-shell preparation is isolated in the following manner. The oyster is placed on its left valve and the hinge is gently pried open with an oyster knife or scalpel. The knife is then twisted in the hinge in order to open the valves slightly, and the adductor muscle is severed in the middle with a razor blade. The opening of the valves should be kept at a minimum during this operation so that the mantle is disturbed as little as possible. Unlike many of the bivalves, the mantles of the oyster are fused along the base of the gills. The right valve is lifted slightly, and beginning at the posterior end and working toward the hinge, the right mantle is snipped free from the left mantle, the gills, and the visceral mass with small scissors. The organs attached to the left mantle can next be removed. One now has the two valves with their attached mantles completely intact. When the dissection is performed with sufficient care both mantles remain attached to the shell and retract only slightly. On being placed in sea water the mantles relax and cover the entire shell surface (Fig. 1).

The mantle-shell preparations were suspended in running sea water by means of a plastic V-shaped clamp with the mantle downward. In this position mucus is eliminated more efficiently.

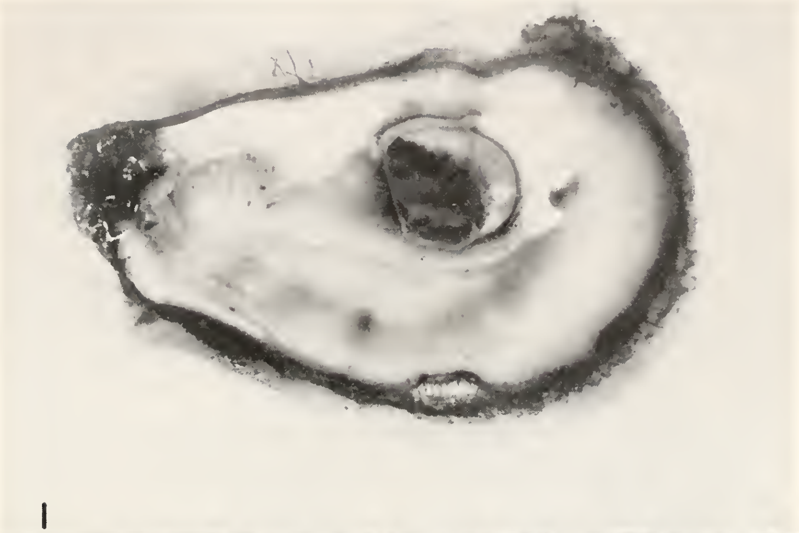
To establish the ability of the mantle to deposit shell *in vitro*, two or three fragments of glass coverslips were inserted between the mantle and the shell following the method of Brooks (1905) for the whole oyster. This can be conveniently performed by lifting a small portion of the mantle edge with a small spatula and inserting a coverslip fragment. Even in the inverted position, the coverslip fragments were firmly pressed against the shell surface by the mantle. The coverslip fragments were removed after the desired interval, thoroughly washed in distilled water to remove dissolved salts, and examined under the light field and polarizing microscopes.

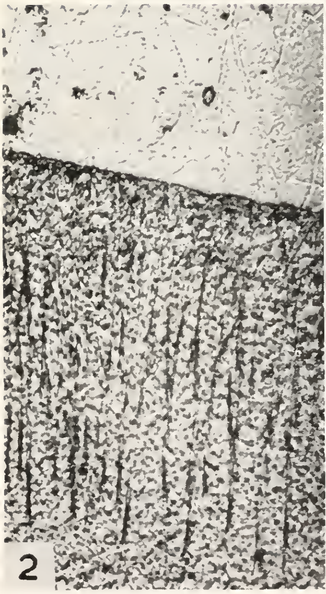
---

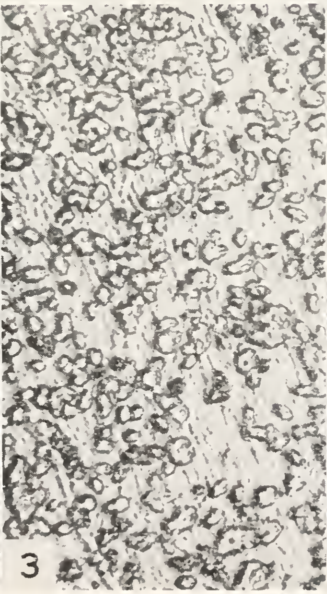
[1] Aided by a grant from the Office of Naval Research.

[2] Present address: Department of Zoology, University of California, Los Angeles 24, California.

[3] Previously *Ostrea virginica*. Re-named by Gunter (1950).







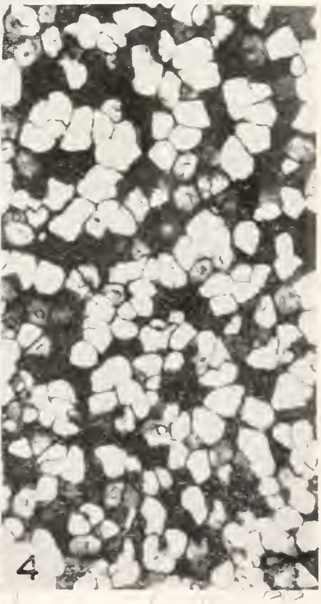


FIGURE 1. Mantle-shell preparation of a right shell.

and magnifications?

FIGURE 2. Area below the diagonal line represents deposits by the mantle-shell preparation on a coverslip fragment over a period of 62 hours. The coverslip was inserted between the mantle and the shell 74 hours after its isolation. The marginal fibrous zone is clearly distinguishable, but the other two zones cannot be easily distinguished without the aid of the polarizing microscope.

FIGURE 3. Crystalline deposits of the innermost zone (structureless). Lower zone of Figure 2 magnified.

FIGURE 4. The zone of honeycomb pattern photographed between crossed Nicol prisms. Intermediate zone of Figure 2 magnified.

These experiments were performed during June and July, 1950. The temperature of the sea water was 26–30 degrees C. and the salinity range was 31 to 36 parts per thousand.

## RESULTS

The mantle-shell preparations remained in good condition for as long as seven days, as indicated by normal ciliary movement and retraction of the mantle edge on mechanical stimulation. More recently, Jodrey (1953) has found the respiration to be normal for the same duration. Longer periods of survival were not investigated.

The examination of pieces of coverslip inserted between the mantle and shell after various intervals revealed that a sheet of organic material was first elaborated followed by the deposition of birefringent crystals. These crystals appeared eight hours after insertion and produced bubbles in dilute acid. These increased in size as well as in number and became contiguous (Fig. 3). Within about 2½ days an organized shell structure began to appear. After three days the deposits showed three main concentric zones of organic material (Fig. 2): (1) a radiating fibrous zone at the outer margin; (2) a zone of honeycomb pattern central to this; and (3) a structureless sheet innermost. The fibrous zone consisted of an organic sheet with radiating fibers running parallel to the surface and perpendicular to the periphery of the mantle. The honeycomb zone showed cellular-like structure probably with open upper surfaces since some of the small areas contained crystals whereas others did not. Except for the absence of fibers the innermost zone resembled the fibrous zone. The fibrous and honeycomb structures were much thicker than the structureless organic sheet and together measured about one mm. in width where oysters of 10 cm. in length were used. The thickness of the organic deposits gradually decreased toward the center of the shell. The three zones resembled the structure of the periostracum, prismatic and nacreous layers of normal shell except that the polyhedral units of the periostracum were lacking.

Birefringent crystalline materials were deposited on all three zones, the most dense being on the honeycomb area. As a result, this region is the most distinct of the three regions when observed between crossed Nicol prisms (Fig. 4). Crystals were imbedded in the cellular framework of the honeycomb region, but were simply scattered on the other two zones. With treatment in 0.001 N HCl the crystalline material dissolved while the organic structures remained intact.

Not all of the preparations were capable of depositing crystals within the 8-hour period mentioned, but pieces of coverslip left in place for more than 40 hours were rarely without crystalline deposits. By introducing the glass fragments at intervals it was found that crystal deposition was possible even after four days of isolation. However, the time required for deposition was longer than with a recently isolated mantle. While birefringent crystals were deposited by all areas of the mantle, the rate of deposition near the muscle was slower than near the margin. This was confirmed later by the use of $Ca^{45}$ (Jodrey, 1953).

## DISCUSSION

Studies of shell formation *in vivo* by Bevelander and Benzer (1948), confirming the observations of earlier workers, showed that the organic matrix is first deposited as a thin sheet and comes to be arranged in prisms and striae in later development. Granules from the mucus cells are deposited in the organic matrix and undergo crystal growth and continue to grow eventually into arrangements whereby the crystals come to be enclosed in a thin layer of organic matrix and assume a polyhedral shape (in *Pinna*).

Bevelander and Martin (1949) reported that isolated strips of mantle of *Pintata radiata* can be maintained *in vitro* and will produce crystals in the course of several days. In the mantle-shell preparation described here the association of mantle and shell of the intact oyster is maintained; and both organic and crystalline shell components are elaborated in patterns closely resembling the normal. This synthetic capacity, together with the stability *in vitro,* emphasizes the utility of the preparation for the study of shell-forming mechanisms of the mantle. The potentialities are still further increased by the use of radioisotopes for quantitative measurements of shell formation (Wilbur and Jodrey, 1952).

The mantle-shell preparation forms shell more slowly than the whole oyster as shown by crystal formation on coverslip fragments inserted beneath the mantle and by the rate of calcium deposition as measured by $Ca^{45}$ (Jodrey, 1953). Other differences may also become evident as shell formation *in vitro* is studied in more detail. Differences between the mantle-shell preparation and the intact oyster must be considered a fortunate circumstance, for by their study and correction through experimental procedures, information concerning factors operative in the formation of shell should result.

The author is indebted to Dr. K. M. Wilbur of Duke University for his advice on the experimental work and critical review of this paper. He also wishes to express his thanks to Dr. W. A. Chipman and Dr. C. E. Atkinson of the U. S. Fish and Wildlife Service, Beaufort, N. C., for providing laboratory facilities and experimental material.

## SUMMARY

1. A preparation consisting of an oyster mantle and its attached shell has been devised for *in vitro* studies of shell formation. Shell consisting of typical organic matrix and birefringent crystalline material was elaborated by this preparation, though at a slower rate than in the intact oyster.

2. All parts of the mantle were shown to be capable of shell formation *in vitro.*

3. The isolated mantle-shell preparation can be maintained in good condition for several days.

## LITERATURE CITED

Bevelander, G., and P. Benzer, 1948. Calcification in marine molluscs. *Biol. Bull.,* **94**: 176–183.

Bevelander, G., and J. Martin, 1949. Culture of mantle tissue of marine molluscs. *Anat. Rec.,* **105**: 614.

Brooks, W. K., 1905. The oyster. Johns Hopkins Press, Baltimore.

Gunter, G., 1950. The generic status of living oysters and the scientific name of the common American species. *Amer. Mid. Nat.,* **43**: 438–449.

Jodrey, L. H., 1953. Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$. *Biol. Bull.,* **104**: 398–407.

Wilbur, K. M., and L. H. Jodrey, 1952. Studies on shell formation. I. Measurement of the rate of shell formation using $Ca^{45}$. *Biol. Bull.,* **103**: 269–276.

# STUDIES ON SHELL FORMATION. III. MEASUREMENT OF CALCIUM DEPOSITION IN SHELL AND CALCIUM TURNOVER IN MANTLE TISSUE USING THE MANTLE-SHELL PREPARATION AND $Ca^{45}$ [1, 2]

LOUISE H. JODREY

*Department of Zoology, Duke University, and Shellfish Laboratory, Fish and Wildlife Service, Beaufort, N. C.*

As a prerequisite to a study of the mechanisms of shell formation, the suitability of the isotope method for measuring shell growth in the intact oyster has been studied and reported in the first paper of this series (Wilbur and Jodrey, 1952). With the use of $Ca^{45}$, shell growth taking place in a few hours could be detected and measured. The isotope method also made possible a study of the distribution of newly deposited calcium on the inner shell surface.

With the radioisotope method for measuring rate of calcium deposition in the whole oyster established, it was desirable as a next step to eliminate some of the variables of the whole oyster and to direct more attention to the mantle itself. Bevelander and Martin (1949) have shown that excised strips of mantle will deposit calcium; and Hirata (1953) has found that a preparation consisting of the intact mantle of the oyster with its attached shell will survive for several days and will deposit both the organic matrix and the calcite of shell. This latter preparation is valuable in that it eliminates the complexity of the whole animal and yet maintains the integrity of the mantle-shell association. The mantle-shell preparation has been studied here with respect to its capacity to deposit calcium, and the rate has been compared with that of the whole oyster.

The mollusc mantle has long been considered as the tissue immediately responsible for the secretion of shell; and it was indeed surprising to find that the $Ca^{45}$ of mantle tissue of whole oysters and mantle-shell preparations in labeled sea water was extremely low, even though a relatively large amount of the isotope was being deposited in the shell. This condition of low activity of the mantle and high activity of the shell was suggestive of two mechanisms. The calcium deposited in the shell might not actually enter the mantle at all; or, a very small amount of calcium might enter the mantle and turn over (be renewed) at a very rapid rate and then become deposited in the shell. To test these hypotheses the turnover rate of calcium in the mantle was studied in the mantle-shell preparation which, in limiting the source and fate of calcium, provides a simpler system than the whole oyster.

---

[1] Aided by a grant from the Atomic Energy Commission.

[2] The author is deeply indebted to Mr. C. E. Atkinson and Dr. W. A. Chipman of the U. S. Fish and Wildlife Service at Beaufort, N. C., who provided laboratory facilities which made this study possible.

## METHODS

### *Rate of calcium deposition in the mantle-shell preparation*

Oysters of the species *Crassostrea virginica* were collected near Beaufort, N. C., during the latter part of June, 1951, and maintained near the laboratory in natural waters. The species, habitat and methods of collection and maintenance of these oysters were the same as those used in immediately preceding experiments on the whole oyster (Wilbur and Jodrey, 1952).

The mantle-shell preparations were prepared as described by Hirata (1953). Each preparation was then placed in one liter of sea water in a large finger bowl and aerated. Some of the mantles retracted during dissection; and after a time when they had again spread out over the shell, $Ca^{45}$ was added to the water. The added isotope was carrier-free in the form of the chloride. The activity was 3.45 microcuries as standardized with a calibrated $Co^{60}$ reference source. The containers were covered with water-proof paper to prevent loss during aeration. The temperature of the water, though nearly constant for each experiment, varied from 25° C. to 28.2° C. during the entire experimental period. The salinity of the water used ranged from 34.84 to 35.61 parts per thousand.

After intervals of 8, 12 and 24 hours, the preparations were removed and the radioactive calcium deposited on the shells was measured as previously described (Wilbur and Jodrey, 1952). In experiments of 24 hours duration the radioactive sea water was changed at the end of 12 hours. Measurements of radioactivity of circular areas of 6.2 $cm.^2$ were made on the anterior, center and posterior regions of the inner shell surface.

### *Calcium turnover rate in the mantle*

For the study of calcium turnover rate in the mantle each mantle-shell preparation was placed in 500 ml. of aerated sea water with a $Ca^{45}$ activity of 5.8 microcuries until the $Ca^{45}$ in the mantle reached equilibrium, *i.e.* the specific activity became maximal. After this period (usually four hours) in radioactive sea water, the preparations were placed in an equal volume of sea water containing no $Ca^{45}$ and allowed to remain there for intervals from 10 to 130 minutes so that the rate of disappearance of $Ca^{45}$ from the mantle could be determined. On removal from sea water the mantle edge[3] was dissected from the remainder of the mantle; both portions were rinsed briefly in sea water, drained for 10 seconds on filter paper, after which each was weighed with a torsion balance and placed in a separate glass counting cup. The tissue was then digested in the same cups with fuming nitric acid and dried with a heat lamp for subsequent measurement of $Ca^{45}$. $Ca^{45}$ deposited on shells was also determined as previously outlined (Wilbur and Jodrey, 1952).

A more complete examination of the uptake of $Ca^{45}$ by the mantle was also carried out by placing mantle-shell preparations in radioactive sea water, removing at intervals of 10 minutes to 8 hours and carrying out the procedure as above.

[3] The term "mantle edge" refers to the thickened, tentacled, pigmented border of the mantle. Because this portion differs macroscopically, histologically and histochemically from the remainder of the mantle, it is not unreasonable to suppose that the two portions have different roles in shell formation which might be reflected in different calcium turnover rates. Hence, the two portions of the mantle were treated separately.

Twelve mantle-shell preparations were used in each experiment. In six experiments the temperature varied from 26.1° C. to 27.9° C., while the salinity range was 35.16 to 35.90 parts per thousand.

Total calcium content of mantle tissue was determined for mantle edge and mantle interior on pooled samples from 16 oysters. The tissue was dry-ashed (at a temperature not high enough to cause loss of Ca) to a white residue which was then dissolved in dilute HCl, diluted to 200 ml. and the calcium determined by a method which excludes magnesium and the alkali metals (Kolthoff and Sandell, 1943).

## Results

### *Calcium deposition in shell*

The shells of the mantle-shell preparations became rapidly radioactive in radioactive sea water, measurable amounts of $Ca^{45}$ being deposited in one hour (not included in graphs). As in the whole oyster, the highest rate of deposition is in the posterior region of the shell. After 24 hours exposure to 3.45 microcuries of $Ca^{45}$, the mean deposition of $Ca^{45}$ in some 20 shells expressed in counts per minute per 6.2 $cm.^2$ of shell was 657 for posterior, 407 for center and 182 for anterior regions. Figure 1 shows the rate of calcium deposition in the posterior region of shell of 37 mantle-shell preparations. As in the whole oyster, a high degree of variability is found in rate of calcium deposition in the mantle-shell preparation also, but the mean rate is very nearly linear.

An approximation of the total amount of calcium deposited after a given period of time on the inner shell surface can be made by comparing the average activity of the whole surface of the shell with the activity of the sea water medium which contains a known amount of calcium. (See Wilbur and Jodrey, 1952, for details and limitations of the method.) An approximation of the average activity of the entire surface can be made by averaging the activity of the three regions measured. The calculated amount of calcium deposited during 24 hours by a mantle-shell preparation of an oyster about 8 cm. long is 1.27 mg. Calculated by the same method, the amount of calcium deposited by the whole oyster was 11.08 mg. for the same period.

### *Calcium turnover rate in the mantle*

Data for the rate of uptake of $Ca^{45}$ by the mantle edge are plotted in Figure 2, in which $Ca^{45}$ per gram of tissue is given in counts per minute as a function of hours of exposure to radioactive sea water. It is apparent that the $Ca^{45}$ content rises very rapidly. The greater part of the uptake is completed after about one-half hour, while the maximum amount of $Ca^{45}$ has been taken up after two to four hours. A comparison of the specific activity of mantle calcium and sea water calcium allows one to calculate the amount of calcium which has been renewed in the mantle during this interval. The method of calculation follows. At the maximum (and equilibrium) the average $Ca^{45}$ activity is about $7 \times 10^2$ counts per minute per gm. wet weight. The calcium content of this weight of mantle edge is 0.95 mg. as determined by analysis for total calcium. Correcting the mantle edge activity to the same geometry used for measurement of sea water activity, the specific activity of the mantle edge calcium is $7 \times 10^{-4}$ microcuries per mg. The specific activity of the sea water calcium is $2.9 \times 10^{-2}$ microcuries per mg. The specific activity of the mantle edge calcium at equilibrium is 2.4 per cent of the specific activity of the sea water which means that 2.4 per cent of the mantle calcium has been renewed. Since it is not possible to further increase the specific activity of the calcium in the mantle, it must be concluded that the greater portion of the mantle calcium is inert and only a small percentage, 2.4 per cent, is being renewed. Since the mantle edge contains 0.95 mg. calcium per gm. wet weight, the portion being renewed is 2.4 per cent of 0.95 mg. or 0.023 mg.

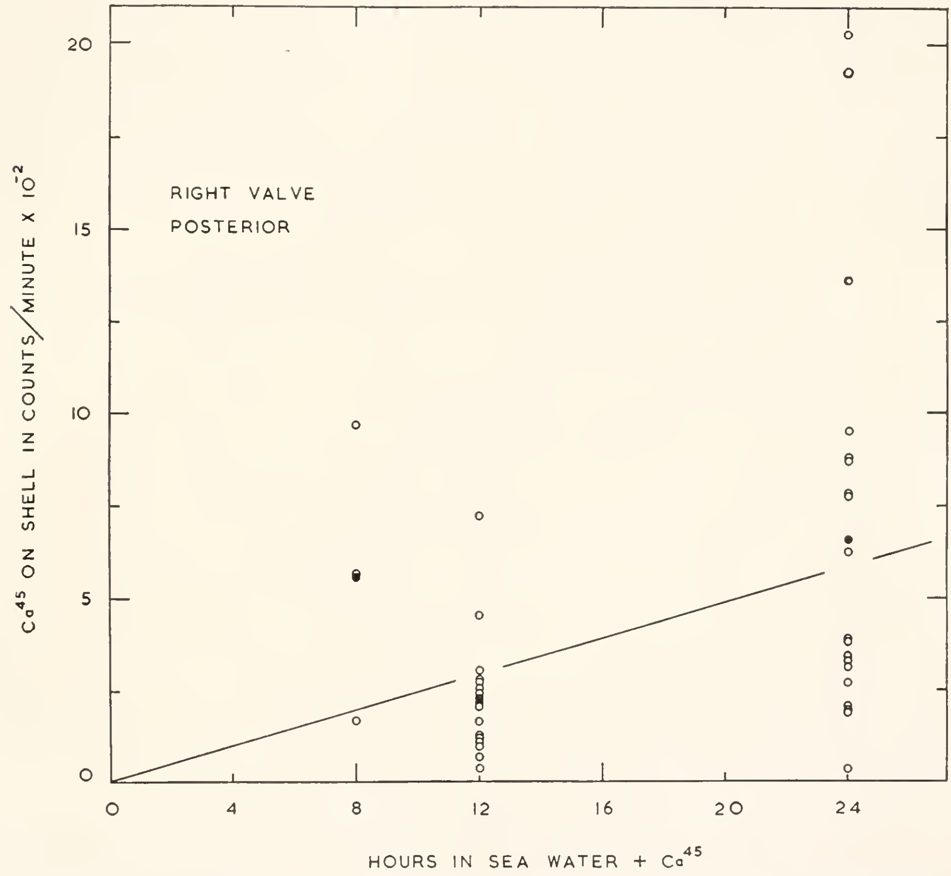


FIGURE 1. Rate of calcium deposition in the mantle-shell preparation. $Ca^{45}$ deposited on a 6.2 cm.$^2$ area in the posterior region of the shell is plotted as a function of hours of exposure to radioactive sea water. (Original counts have been multiplied by $10^{-2}$ in order to use a one-digit scale.) Each point represents a measurement on a different individual. The mean rate is very nearly linear.

$Ca^{45}$ uptake by the mantle interior follows much the same pattern as shown in Figure 2, but the $Ca^{45}$ content at equilibrium is about one-half as great as that in the mantle edge. Since there is only about one-half as much total calcium in this part of the mantle, the specific activity is therefore the same as in the mantle edge. The figure of 2.4 per cent for the actively participating calcium accordingly holds for mantle interior also.

Disappearance of $Ca^{45}$ from the mantle edge is extremely rapid (Fig. 3), the greater fraction being removed during the first half hour, indicating a very high rate for renewal of calcium. The disappearance of calcium from the mantle interior is also a rapid process, the accumulated radioactive calcium leaving the mantle interior during the same time interval.

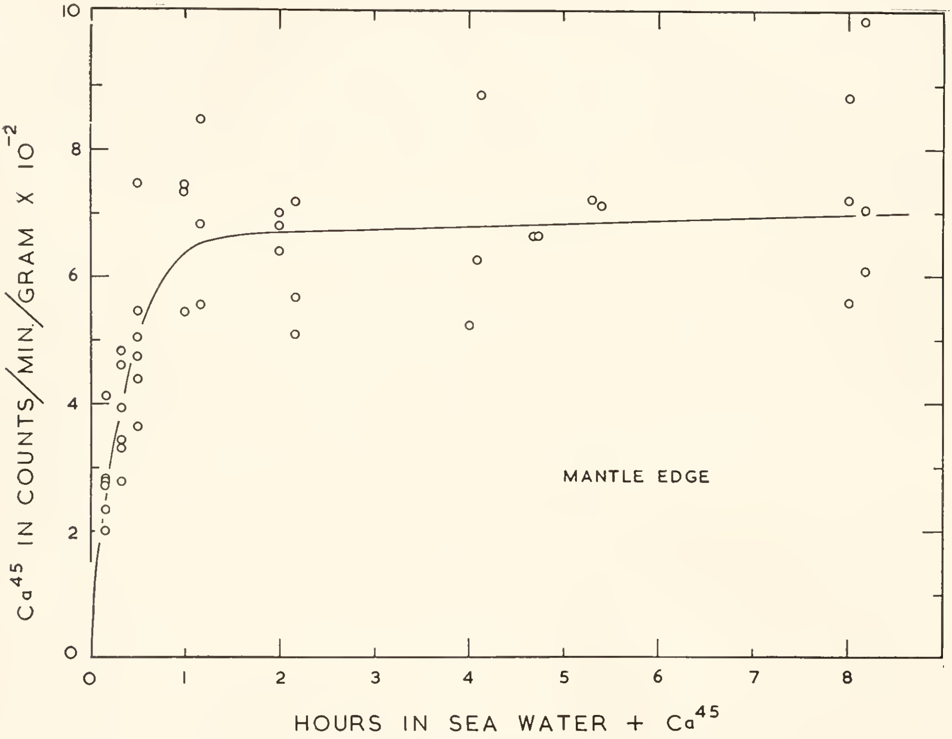


FIGURE 2. Rate of accumulation of $Ca^{45}$ in the mantle edge. The amount of $Ca^{45}$ in the mantle edge in counts per minute per gram wet weight is given as a function of time in radioactive sea water. Each point represents a measurement of $Ca^{45}$ in an individual mantle. Most of the uptake is completed after one-half hour. After two to four hours the mantle has taken up as much $Ca^{45}$ as it will accumulate.

The rates of disappearance of $Ca^{45}$ from mantle edge and mantle interior are exponential functions. From a semi-log plot of the data (Fig. 4) the half-time (period of time required for one-half the $Ca^{45}$ to leave the mantle) is found to be 16.5 minutes. The turnover time (see Hevesy, 1948 and Siri, 1949) is 16.5/0.693 or 24 minutes. The turnover rate, which is the actual weight of calcium disappearing and being replaced per unit time, is 0.023/24 or $1 \times 10^{-3}$ mg. per minute. In 24 hours the calcium turnover in one gram of mantle edge would be 1.44 mg. A similar calculation gives a calcium turnover of 0.62 mg. per gram of mantle interior. The complete mantle of one oyster of the size used weighs about 1.5 grams, the mantle edge comprising about 0.5 grams of the total. Hence the calcium renewed per day in the whole mantle would be $1.44/2 + 0.62$ or 1.34 mg. The estimated amount of calcium deposited on shell in the same period as measured from

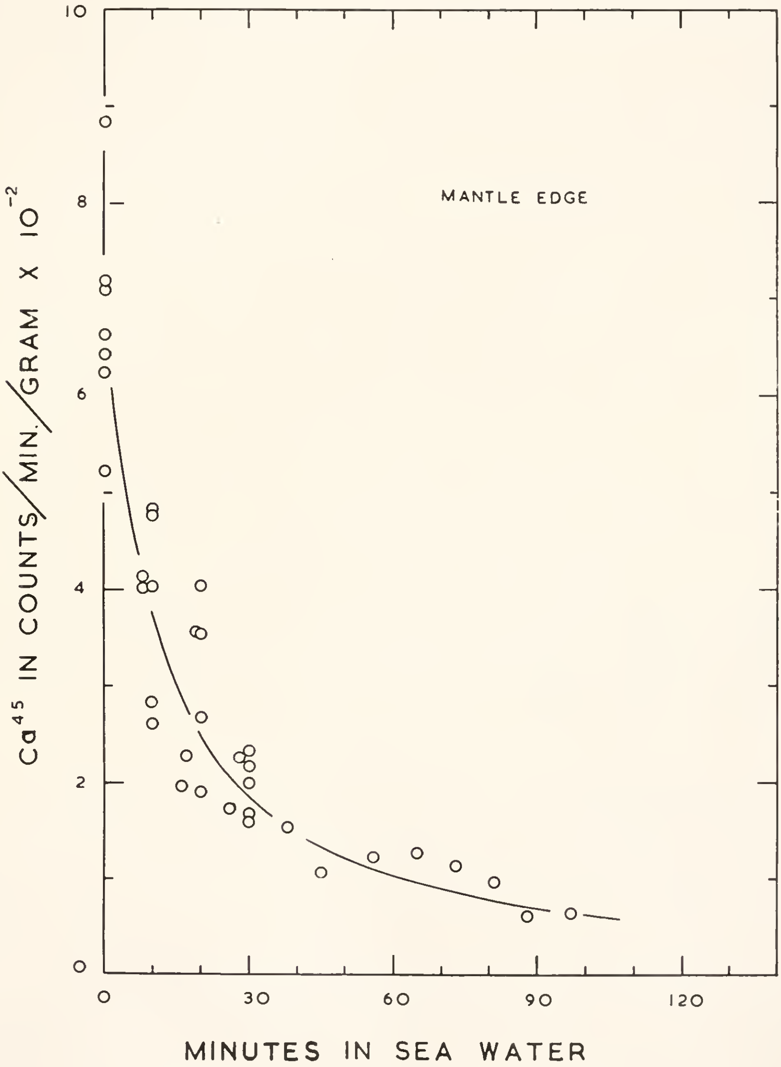


FIGURE 3. Rate of disappearance of $Ca^{45}$ from the mantle edge when placed in non-radioactive sea water. After the mantles had taken up as much $Ca^{45}$ as they would accumulate, they were placed in non-radioactive sea water and the rate of loss of the isotope from the mantle was determined by measuring the $Ca^{45}$ content of the mantle after increasing intervals. Each point is for a separate mantle. The rate of disappearance is very rapid; most of the $Ca^{45}$ has disappeared after one-half hour. Although individual variability exists, the curve is an exponential function with time.

counts of radioactivity of shell was 1.27 mg. This figure was calculated from the average activity of the inner shell surface, the rate of calcium deposition being lower in the central region of the shell which is in keeping with the lower calcium turnover rate of the mantle interior.

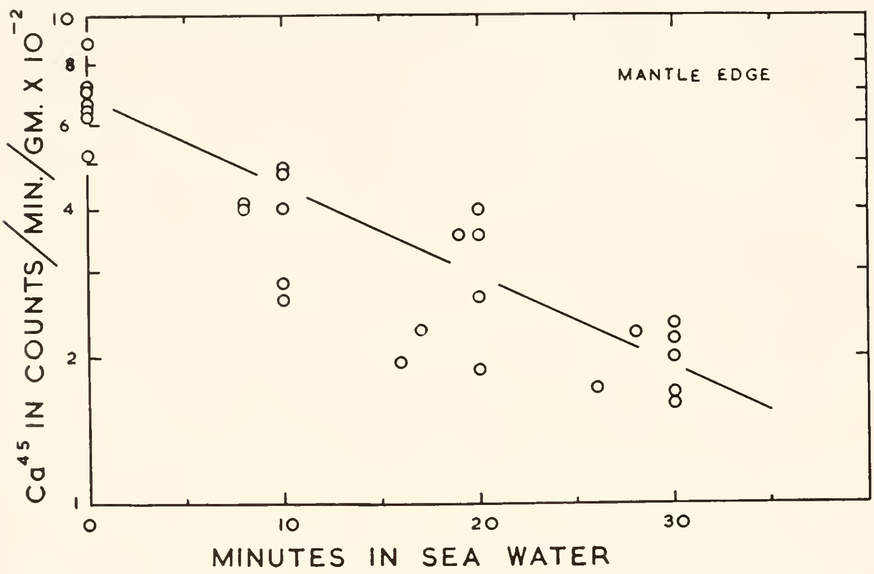


FIGURE 4. Semi-log plot of rate of disappearance of $Ca^{45}$ from mantle edge. $Ca^{45}$ in counts per minute per gram of mantle tissue is plotted on the logarithmic scale and time is represented by an arithmetic scale. From this straight line curve the turnover time for calcium in the mantle can be determined as described in the text. The turnover time for the calcium being renewed is about 24 minutes.

## DISCUSSION

The mantle-shell preparation, consisting of a right valve with its attached mantle, is extremely valuable for the study of shell formation. One might compare the mantle-shell preparation for the study of shell formation to the use of tissue slices for metabolism studies, with the former having the advantages of remaining alive for several days and being an intact system with respect to the relationship between mantle and shell. Because of the viability of the tissue and its ability to deposit shell, the mantle-shell preparation provides a convenient system for studying shell formation under a variety of experimental conditions, especially when combined with the use of radioactive calcium. The effects of various inhibitors and respiratory substrates on the rate of shell formation will be studied and reported in another paper in this series. Since this preparation limits the source and fate of mantle calcium, its use, combined with the isotope technique, allows one to study directly the path taken by calcium which becomes deposited as shell.

This present study has shown that the mantle of the mantle-shell preparation will deposit as shell substance calcium from the surrounding sea water. Experimental evidence that the calcium of shell enters the mantle directly from the sea water has not been at hand, although suggestions that it may do so have been made (Galtsoff, 1934; Robertson, 1941). Apparently, at least part of the calcium deposited as shell does not require the participation of digestive and circulatory systems; although Bevelander (1952), reporting on uptake of $Ca^{45}$ by intact molluscs, states that calcium ions present in the water are ingested by the organism and are localized in several organs. Although an exact comparison of rates of calcium deposition in the whole oyster (Wilbur and Jodrey, 1952) and the mantle-shell preparation cannot be made because the latter work was done about a month later in the season, and at a different temperature, it is clear that the isolated mantle deposits calcium at a much lower rate. Neglecting discrepancies which may enter due to season (Loosanoff and Nomejko, 1949), the mantle-shell preparation deposits calcium only about one-ninth as rapidly as the whole oyster. The pattern of deposition, however, is very nearly the same; and it seems reasonable to assume that the usual shell-forming mechanisms are present but operating at a lower rate (see Hirata, 1953). Whether the decreased rate is due to lack of a more efficient transport system for calcium or to a diminished energy supply is not known. Possibly an adequate source of energy might make the rate of calcium deposition in the mantle-shell preparation more nearly comparable with that of the intact animal. This assumption will be tested by adding to the medium respiratory substrates which are known to affect mantle respiration (Hilgartner, 1951; Jodrey and Wilbur, unpublished data) and determining their effect on calcium deposition.

In the introduction it was mentioned that the low $Ca^{45}$ content of the mantle tissue and high isotope content of the shell could be made compatible by one of two mechanisms: either the calcium deposited as shell did not actually enter the mantle cells or a very small amount of calcium in the mantle was turning over at a very rapid rate. The results obtained in this study of turnover rate support the second supposition. By a comparison of specific activity of mantle calcium with the specific activity of sea water calcium, it was shown that only about 2.4 per cent of the mantle calcium is actually being renewed under the experimental conditions described. The remainder is inert and does not move out of the mantle. The amount of calcium renewed in the mantle during a 24-hour period would be about 1.34 mg., while the amount of calcium deposited on shell was calculated to be about 1.27 mg. It would seem from these two figures that in the mantle-shell preparation at least, the greater part of the calcium being renewed in the mantle is being deposited as shell, rather than exchanging with the sea water. It seems reasonable to assume that this situation would also obtain in the whole oyster. Thus, in spite of the relatively high calcium content of the mantle (2.5 times that in sea water), shell deposition is apparently brought about by a small portion turning over at a very rapid rate. Whether this would be true also for the whole oyster cannot be said, although the consistently small amounts of $Ca^{45}$ found in mantles of intact oysters depositing relatively large amounts of the isotope in the shell would indeed point to this.

The large amount of calcium in the mantle which appears to be inert is of considerable interest. Molluscan mantles have been observed by several workers to contain granules of calcium phosphate (Hayasi, 1939; Biedermann, 1914; Bevelander and Benzer, 1948) or conglomerates of calcium and organic material (Hayasi, 1938). These large granules may perhaps constitute the inert portion.

Under certain conditions of the whole oyster, such as long periods of closure when the acidity of body fluids increases (Dugal, 1939), this calcium may be mobilized. This could be tested experimentally by an examination of the mantle for granules after periods of forced closure. Combined autoradiographic and histochemical studies of mantle sections might also aid in a more accurate localization of inert and dynamic calcium.

The author wishes to thank Dr. Karl M. Wilbur, whose suggestions and criticisms have been most helpful during both the experimental work and preparation of the manuscript.

## SUMMARY

1. A study of calcium turnover and calcium deposition by the oyster mantle in the absence of other soft tissues has been carried out using mantle-shell preparations of *Crassostrea virginica*.

2. The mantle of the mantle-shell preparation is able to deposit calcium from the sea water in shell. At least part of the calcium deposited as shell substance does not require the participation of digestive and circulatory systems.

3. The rate of deposition in the mantle-shell preparation was about one-ninth that of the whole oyster under the conditions of the experiment, but the distribution of newly deposited calcium was similar in both.

4. The greater portion of the calcium in the mantle appears to be inert, since the specific activity of the mantle calcium could not be increased beyond a small percentage of the specific activity of the sea water calcium. A small fraction (2.4 per cent) was renewed every 24 minutes, the turnover rate being 0.6 micrograms of calcium per minute per gm. of mantle.

5. The calcium turnover rate in the mantle edge is approximately twice as rapid as in the mantle interior but since the latter is renewing a calcium reservoir which is only about one-half as great as the former, the turnover time for the renewing fraction is the same in each.

6. Since the amount of calcium being renewed in the mantle is very nearly the same as the amount being deposited on the shell, it would seem that in the mantle-shell preparation at least, the fraction of rapid turnover brings about the formation of shell.

## LITERATURE CITED

BEVELANDER, G., 1952. Calcification in molluscs. III. Intake and deposition of $Ca^{45}$ and $P^{32}$ in relation to shell formation. *Biol. Bull.*, **102**: 9–15.

BEVELANDER, G., AND P. BENZER, 1948. Calcification in marine molluscs. *Biol. Bull.*, **94**: 176–183.

BEVELANDER, G., AND J. MARTIN, 1949. Culture of mantle tissue of marine molluscs. *Anat. Rec.*, **105**: 614.

BIEDERMANN, W., 1914. Physiologie der Stutz und Skelettsubstanzen. Handbuch d. vergl. Physiol. Hans Winterstein, 3 Bd., 1 Halfte. Jena.

DUGAL, L. P., 1939. The use of the calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. *J. Cell. Comp. Physiol.*, **13**: 235–251.

GALTSOFF, P. S., 1934. The biochemistry of the invertebrates of the sea. *Ecol. Monogr.*, **4**: 481–490.

HAYASI, K., 1938. Detection of calcium in molluscan mantles. I. Anodonta and Christaria. *Annot. Zool. Japon.*, **17**: 95–103.

Hayasi, K., 1939. Detection of calcium in molluscan mantles. II. *Annot. Zool. Japon.*, **18**: 1–7.

Hevesy, G., 1948. Radioactive indicators. Interscience Publishers, Inc., New York.

Hilgartner, Margaret W., 1951. Investigations on the presence of the Krebs tricarboxylic acid cycle in mantle tissue of *Ostrea virginia*. Master's thesis. Duke University.

Hirata, A. A., 1953. Studies on shell formation. II. A mantle-shell preparation for in vitro studies. *Biol. Bull.*, **104**: 394–397.

Kolthoff, I. M., and E. B. Sandell, 1943. Quantitative inorganic analysis. The Macmillan Co., New York.

Loosanoff, V. L., and Charles A. Nomejko, 1949. Growth of oysters, *O. virginica* during different months. *Biol. Bull.*, **97**: 82–94.

Robertson, J. D., 1941. The functions and metabolism of calcium in the invertebrates. *Biol. Rev.*, **16**: 106–133.

Siri, W. E., 1949. Isotopic tracers and nuclear radiations. McGraw-Hill Book Co., Inc., New York.

Wilbur, K. M., and L. H. Jodrey, 1952. Studies on shell formation. I. Measurement of the rate of shell formation using Ca$^{45}$. *Biol. Bull.*, **103**: 269–276.

# THE FIBRILLAR SYSTEMS OF CILIATES AS REVEALED BY THE ELECTRON MICROSCOPE. I. PARAMECIUM[1]

CHARLES B. METZ, DOROTHY R. PITELKA AND JANE A. WESTFALL

*Dept. of Zoology, University of California, Berkeley 4, California, and Dept. of Zoology, University of North Carolina, Chapel Hill, N. C.*

For many years the fibrillar systems and associated organelles of protozoa have attracted the attention of cytologists and protozoologists. Initially this interest appears to have been directed by morphological and functional considerations. More recently and with the appreciation of the self-duplicating nature of these systems, their causal relation to morphogenesis, regeneration and the polarity of the organism has commanded first attention (Tartar, 1941; Fauré-Fremiet, 1948; Lwoff, 1950; Weisz, 1951). Unfortunately, these studies have been limited by the fact that the structures in question have dimensions which border on the resolving power of the light microscope. Thus, with the exception of cilia, flagella and exploded trichocysts (Jakus and Hall, 1946; Pitelka, 1949; Finley, 1951, and others) the detailed anatomy of the fibrillar apparatus and associated organelles has remained obscure. For an adequate understanding of the functional and morphogenetic properties of these systems a detailed picture of their fine anatomy would appear to be essential. Presumably such a picture could be obtained by an electron microscope examination of these systems in serial sections (Sedar, 1952) or preferably after appropriate dissection of the material. In the present study "sonic dissection" was found to be a simple and effective method of preparing fragments of the ciliate protozoan surface, together with the subjacent fibrillar apparatus, for electron microscope examination. The present effort, the first in a series of morphological studies employing this technique, should now provide a new point of departure for the investigation of the physiology of locomotion, coordination and developmental mechanics in ciliate protozoa.

The primary fibrillar apparatus of ciliates consists of a number of parallel structural units, the kineties (terminology of Lwoff, 1950). Each of the several kineties in turn is composed of: 1) a longitudinal row of cilia; 2) a longitudinal row of small granules, one granule at the base of each cilium (the ciliary basal bodies or kinetosomes); and 3) a longitudinal ectoplasmic fiber which connects all the basal granules in the row (the silver line or neuromotor fiber, neuroneme, or kinetodesma). As revealed by the light microscope, this unit of organization is an anatomically continuous structure. In the more complex ciliates this fibrillar pattern is rendered more involved by modifications in the arrangement of the kineties, especially in the oral region, and by superposition of accessory organelles and fibers upon this system of kineties. In the most extensively studied ciliate, Paramecium, this complexity is evident. At least two other ectoplasmic fibrillar systems besides the primary one have been described and the detailed relationships of these are disputed (see Taylor, 1941, for review of fibrillar systems in Paramecium and other ciliates). In the oral region the arrangement of fibers and cilia becomes highly complex (Lund, 1933).

---

[1] This investigation was supported by grants from the National Institutes of Health, U. S. Public Health Service and the American Cancer Society.

Most of the structures described by others using classical methods have been observed in the present study and their relationships have been clarified. However, attention has been directed mainly to the structure and organization of the primary fibrillar apparatus or kineties, with their component cilia, kinetosomes and kinetodesmas. The last are never found to be single "fibers" in the classical sense. As reported briefly elsewhere (Pitelka and Metz, 1952) a kinetodesma is seen to be a bundle of tapering fibrils, each arising independently from a kinetosome and extending for a modest distance along the bundle.

## Materials and Methods

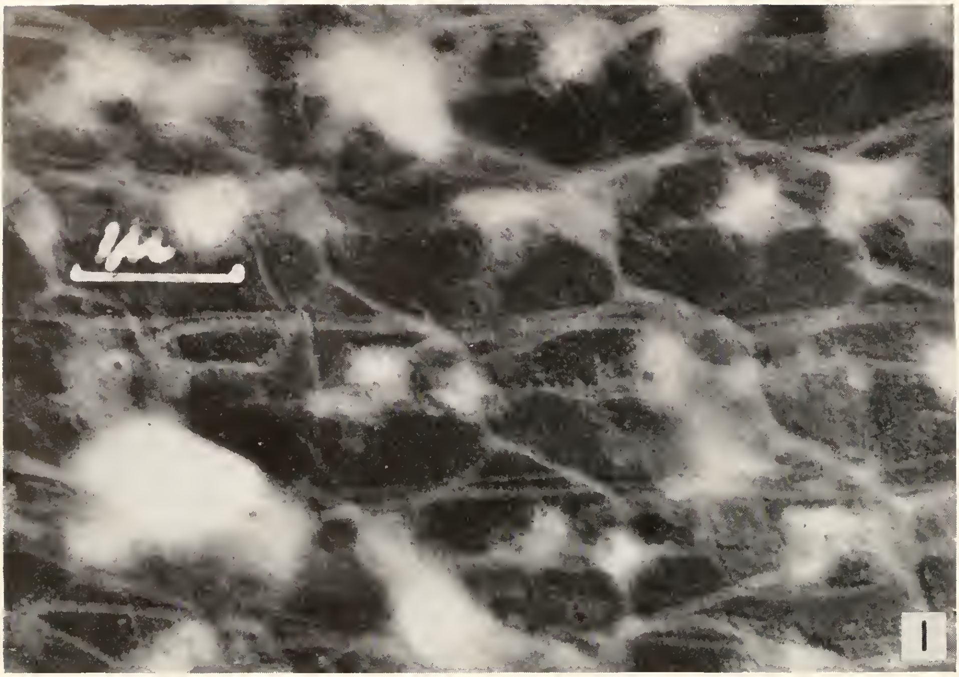
### *Living material and culture conditions*

The following four species of Paramecium were examined in this study: *P. aurelia* (Sonneborn's stock 47, variety 4), *P. multimicronucleatum* (from Kirby's laboratory), *P. caudatum* (from Kirby's laboratory) and *P. calkinsi* (originally from Woodruff's collection). The first three of these were cultured in baked lettuce infusion medium (Sonneborn, 1950) which had previously been inoculated with *Aerobacter aerogenes*. *P. calkinsi* was cultured in identical fashion except that sea water diluted to 2/5 with distilled water was substituted for the distilled water and the $CaCO_3$ was omitted from Sonneborn's formula. With but one or two exceptions only cultures of starved, non-dividing animals were used in the study.

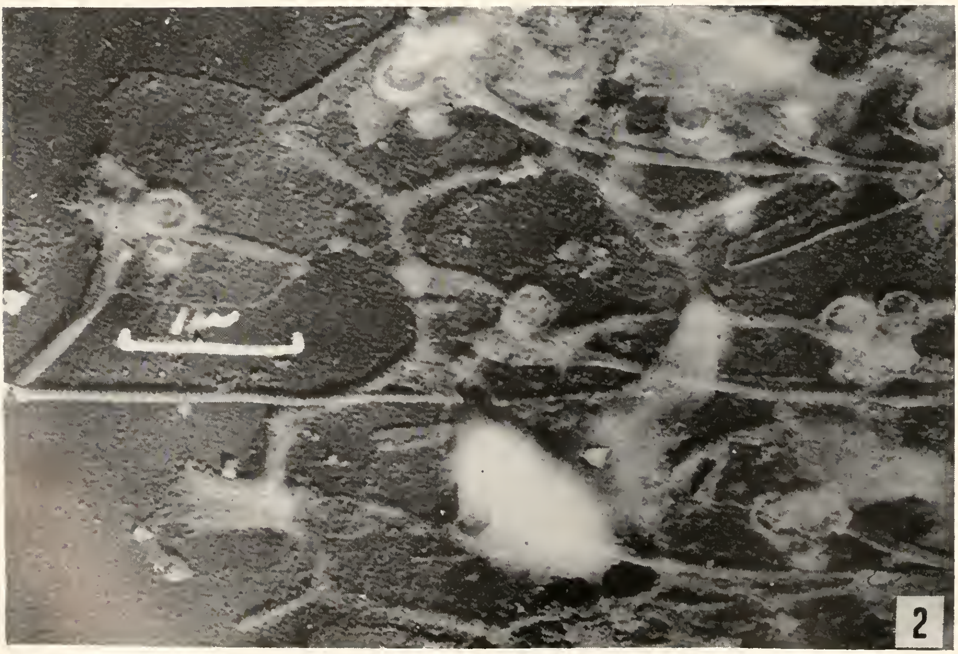
### *Preparation of the material for electron microscopy*

As starting material for the preparation of pellicular fragments rich flourishing cultures of 100 to 300 ml. volume proved to be most satisfactory. When necessary, such cultures were filtered through a loose layer of cotton to clarify the suspension. The culture was then mixed rapidly with sufficient full strength formalin (40% formaldehyde) to give a final formalin concentration of 1 to 2%. The formalin-treated culture was allowed to stand for one to several hours during which time the fixed paramecia settled to the bottom of the vessel. At the end of this period of fixation, the supernatant was removed by aspiration and the concentrated sample of fixed animals was transferred to a 15-ml. centrifuge tube. The procedure employed here has been described in greater detail by Metz (1947) in an unrelated study.

Following fixation the paramecia were washed three times with distilled water (15-ml. washings with mild centrifugation) and finally suspended in 3-5 ml. of distilled water. To prepare the fragments of pellicle this final distilled water suspension was transferred to the receiving cup of a 9 Kc magnetostriction oscillator (Model S–102A, Raytheon Corp.) and vibrated for 30 seconds to several minutes. The duration of this treatment was governed by the degree of dissection desired and the fragility of the individual sample. Some variation was observed in the length of treatment required to break up the animals in different samples. This probably resulted from variations in the fixation procedure. To control these fac-



1



2

tors samples of the suspensions were removed from the receiving cup at 30-second to one-minute intervals and examined with the phase microscope. When the desired degree of disruption had been achieved, the treatment was terminated.

One- to 3-minute treatment usually resulted in thorough disruption of the paramecia. The cilia were stripped from the animals, the pellicles were broken into small fragments and the various formed elements of the endoplasm were released into suspension. Macronuclei, mitochondria and trichocysts in various stages of explosion were frequently observed. Since the present study concerned only the pellicular fragments and attached fibers, the other interesting structures were usually treated as undesirable contaminants and were separated from the pellicular fragments by differential centrifugation insofar as possible. At each step in the procedure the material was examined with the phase microscope. This was found to be necessary to prepare concentrated and relatively pure suspensions of fragments. Small drops of such suspensions were air-dried to collodion-coated electron microscope grids, shadow cast with palladium (*P. calkinsi, P. multimicronucleatum*) or chromium (*P. aurelia, P. caudatum*) and finally examined with the electron microscope (R.C.A. Universal).

### *Limitations*

It is apparent that this method of preparation has certain inherent hazards and limitations. The first of these is the usual danger of introducing artifacts by fixation and drying. To minimize this possible source of error, the material was examined routinely with phase optics in the course of preparation and these observations overlapped those with the electron microscope. As a further precaution the fixation procedure was varied by using osmium tetroxide (osmic acid) vapor instead of formalin in a few instances. Within these limits no radical variation was observed in the preparations.

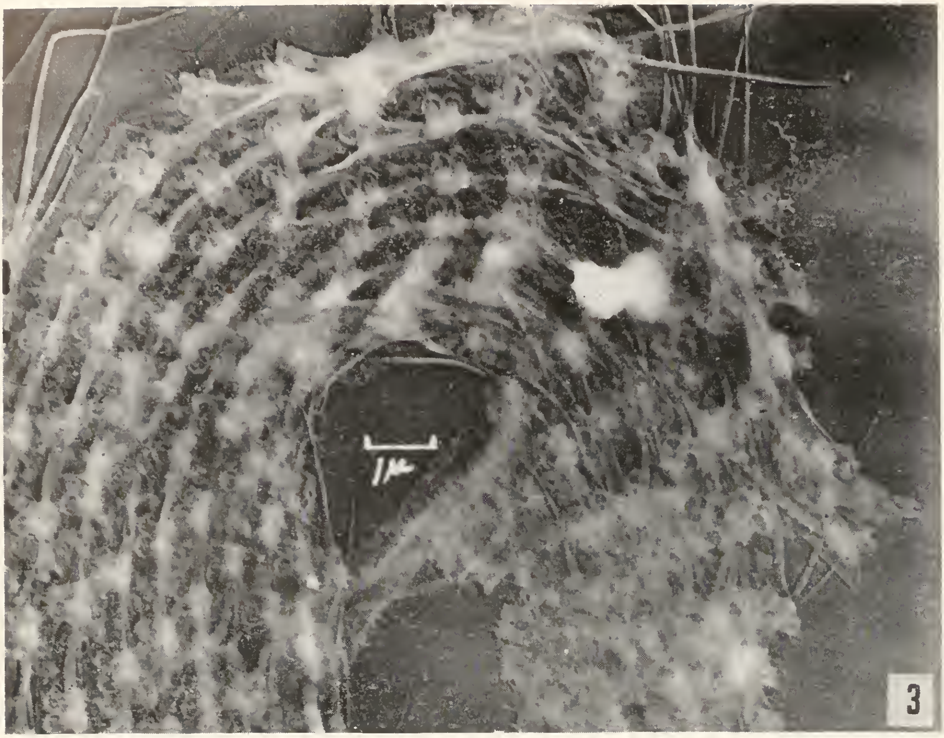
Sonic dissection as used here imposes further limitations on the study. Large numbers of animals were broken up together and fragments were selected for study on a chance basis. No systematic study of a single animal was attempted. To attempt to reconstruct a single organism by direct comparison of such fragments is hazardous and especially so since specific points of orientation are usually lacking in the fragments.

Finally, failure to find a particular structure or structures in these preparations cannot be considered immediate and clear proof of their non-existence in the intact organism, since such structures may have been removed or destroyed by the sonic treatment. The extraordinary feature of the preparations is that so many structures do remain intact.

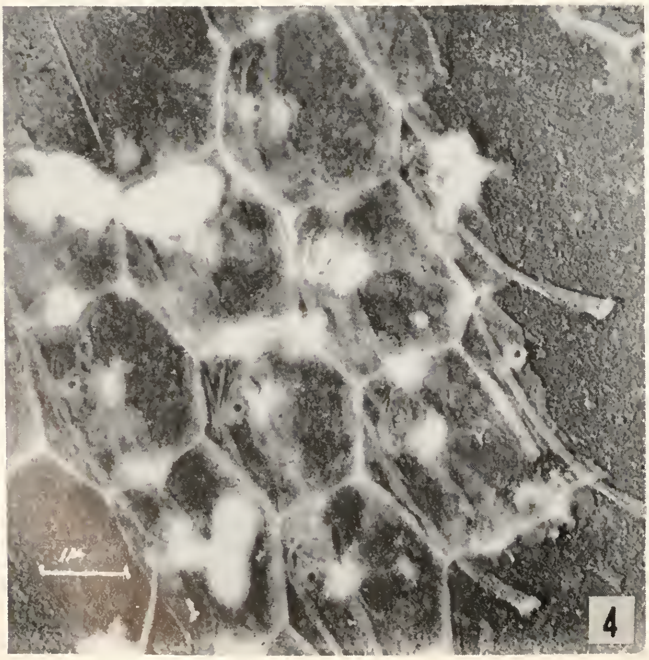
---

FIGURE 1. *Paramecium aurelia.* A fragment of the pellicle viewed from outside, showing the polygon lattice in the form of hexagons and the subpellicular bundles of kinetodesmal fibrils. Approximately 21,600 ×.

FIGURE 2. *Paramecium aurelia.* A fragment of the pellicle viewed from inside the animal. Remnants of trichocysts are attached to the polygon cross bars (lower left). Single and double (right center) ciliary rings and accessory rings (upper left) appear in the centers of the polygons. Some of the ciliary rings are clearly plugged with cross sectional segments of cilia. Ciliary basal stumps and kinetosomes have disintegrated but the kinetodesmal fibrils remain intact. Approximately 24,000 ×.



3



4

5

The reported dimensions of structures and magnification scales must be considered as approximations, since the electron microscopes were not calibrated at regular intervals during the study.

## RESULTS

This descriptive account of the fibrillar systems in Paramecium is based largely on *P. aurelia* and *P. multimicronucleatum*. These two forms have been examined in greatest detail and the study is equally complete for both with the exception that no figures of the oral region of *P. multimicronucleatum* have yet been obtained. Since the two species were not found to differ, the account will apply to both forms.

Figures 1–4 show the type of specimen obtained when the material is properly treated with sonics. The first noticeable feature of the preparations is that the pellicular fragments are nearly free of cytoplasmic material. This might be expected since the pellicles of ciliates may be extracellular membranes (Beams and King, 1941). The really striking feature of the preparations is the collection of fibers that regularly remains attached to these pellicular fragments in spite of the sonic treatment. Indeed, with near minimal treatment, the cilia also remain attached (Figs. 6 and 7). Upon study of Figures 1, 2 and 4 it will be noted that the various structures in the specimens fall into two groups: A) the pellicle and an associated fiber-like lattice and B) a subpellicular fiber system. These are most conveniently considered separately.

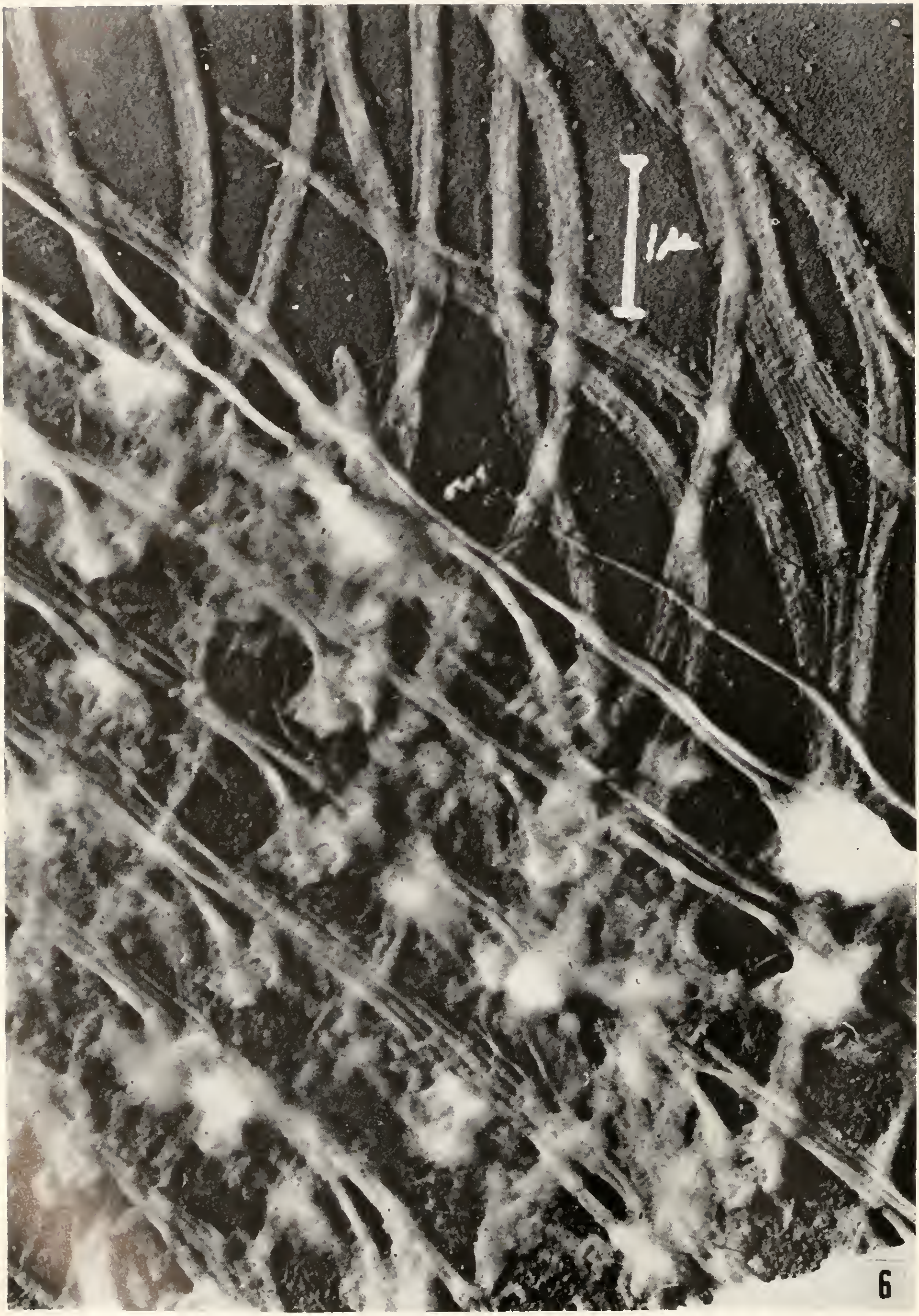
### A. The pellicle and pellicular lattice

As seen in Figures 2 and 4 the pellicle proper is a thin membrane. No ultrastructure is evident in this membrane at the magnifications employed. Intimately associated with the membrane is a fiber-like polygonal lattice. The polygons of this lattice usually assume the form of hexagons or quadrilaterals. In some preparations hexagons, quadrilaterals and intermediate forms are all present. With phase optics the polygon lattice appears as a system of ridges. The centers of the polygons are depressed. This fiber-like lattice is certainly the outer fibrillar system figured by numerous authors. The lattice appears as a continuous structure in all electron photomicrographs. No subfibrils or discontinuities are evident. Sedar (1952), however, reports that the material making up the lattice is compound in structure. The only modification in this structural uniformity is found on the cross bars of the lattice. At or near the middle of the cross bars, varying amounts of material remain attached (Figs. 1, 2, 4). In this region the lattice does not dissect clean. This position is described as the trichocyst attachment

FIGURE 3. *Paramecium aurelia.* A circumoral fragment viewed from inside the animal. The ciliary rings and kinetosomes are paired. A single kinetodesmal fibril arises from each kinetosome pair. Fibrils run in an anterior direction. Those on the left side curve to the right in the preoral region. Approximately 9800 ×.

FIGURE 4. *Paramecium multimicronucleatum.* Fragment viewed from outside. Cones with pitted centers occur in the pellicle. These probably are the accessory rings (Fig. 2) seen from the outside. Several kinetodesmal fibrils are not anchored to kinetosomes, yet they remain attached to the kinetodesmal bundle. Approximately 12,000 ×.

FIGURE 5. *Paramecium calkinsi.* An individual kinetodesmal fibril showing spiral organization. Approximately 48,000 ×.



6

point by most earlier workers (*i.e.*, Lund, 1933; Sedar, 1952), and is so regarded here. However, it should be noted that in no case was an intact, undischarged trichocyst found attached to a pellicular fragment. In Figure 2 a rather substantial amount of material is attached to one cross bar of the lower left polygon. This may be part of an undischarged trichocyst. The suggestion of a fine fibrillar organization in the structure is of some interest.

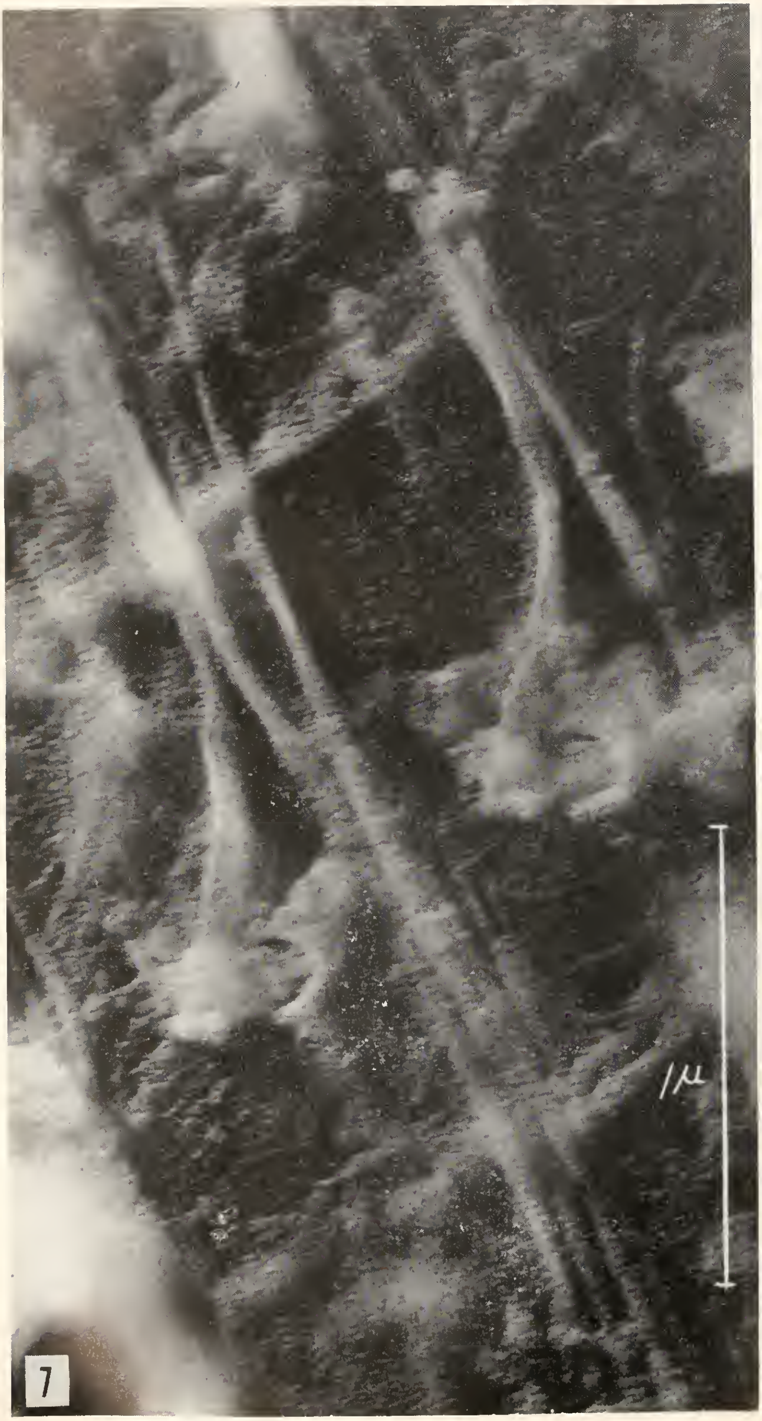
The physical relationship between the outer lattice and the pellicle is disputed. Some investigators have held that the lattice is actually a thickened part of the membrane itself, whereas others (most recently Sedar, 1952) maintain that the membrane and lattice are separate structures. Unfortunately, this study does not clarify the issue materially. However, the lattice and the membrane appear to behave as a unit. No specimens were found in which the lattice and membrane were clearly separated. Furthermore, when the pellicle is torn the line of rupture frequently cuts across both membrane and lattice. Therefore, if these are separate structures rather than a continuous one, they must be cemented together rather firmly.

The cilium is generally stated to pass through the depressed center of the pellicular polygon. This view is in agreement with the present findings. Viewed from the inside of the pellicle (Figs. 6, 7) the cilium is clearly seen to pass through a ring-shaped structure. This is believed to be a thickening in the pellicular membrane. Occasionally (Fig. 2) two such rings occur in a single polygon. This situation appears to be constant in fragments from the circumoral area and agrees with the observations of J. von Gelei (1925, 1934a) that two cilia per polygon is a characteristic feature of this region in *P. nephridiatum* and *P. caudatum*. However, Lund (1933) finds only one cilium per polygon in the circumoral region of *P. multimicronucleatum*. It should be recalled that the oral region of *P. multimicronucleatum* was not examined in the present study.

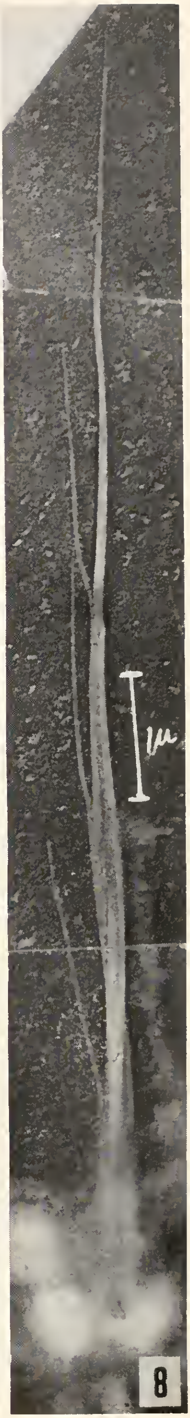
In many preparations a second, smaller ring-shaped structure is found near the ciliary ring. This is seen most readily on an inside view of the pellicle (Fig. 2, upper left). The smaller ring, when present, is constant in position and occurs regularly in many if not all of the polygons in the specimen. The interpretation of outside views in terms of the ciliary ring and the accessory ring is more difficult. In Figure 4 interesting cones with pitted centers are evident. These could be either the external openings of the ciliary rings or the external parts of the secondary rings. The latter possibility is accepted because 1) these cones are very eccentric in position, 2) they do not appear to be associated with the kinetodesmal fibrils to be discussed in the next section, and 3) the opaque structures near the centers of the polygons correspond more nearly to the ciliary rings. These opaque structures are more central in position and larger than the cones; they are associated with kinetodesmal fibrils and finally they occasionally have a suggestion of a larger ring structure. The current investigation gives no hint of a possible function for these secondary rings.

One other structure associated with the pellicle is evident in some preparations. This is a strand or fiber extending across the polygon from one trichocyst attach-

FIGURE 6. *Paramecium aurelia*. Inside view of fragment. Cilia are seen to pass through the ciliary rings in the pellicle and end at the kinetosomes. Kinetodesmal fibrils pass from the kinetosomes and join the kinetodesmal bundles. Approximately 21,000 ×.



7



8

ment point to the ciliary ring and on to the next trichocyst attachment point. Such structures are seen in Figures 1 and 2. The nature of these is not clear. They appear to be fibers of the same sort and continuous with the lattice fibers in Figure 2. However, they are not present in all preparations, particularly those subjected to prolonged sonic treatment, and their form is somewhat variable (Fig. 1). If this material should take a silver stain, it could well have been confused with the primary fibrillar system to be discussed next.

### B. The primary fibrillar or kinety system

Although the pellicle and the outer lattice are not without interest, the primary system is the striking feature of this study. The essentials of this are clearly illustrated in Figure 6 and consist of the three following parts: 1) the cilium which passes from the exterior through the ciliary ring in the pellicle and extends a short distance into the interior, 2) a bulb or knob at the internal terminus of the cilium, and 3) a long tapering fibril that arises from this knob and joins others of the same sort to form a bundle. This bundle runs parallel to both the surface of the pellicle and the rows of cilia.

#### *The cilium*

The cilium itself requires little comment. Its structure, as observed here, agrees with the descriptions of other workers. Thus, the free shaft of the cilium is seen to contain a number of fine longitudinal fibrils and from the studies of Metz and Pitelka (Metz, 1953) it is known to possess a limiting membrane. Unfortunately no clear picture of the proximal origin of the intraciliary fibrils has been obtained in this study although there are indications (Fig. 7) that these fibrils extend to the basal knob. In Figure 2 some of the ciliary rings in the pellicle appear to contain structures that correspond to cross sectional views of the cilium with the contained fibrils. It should be noted that the cilium is never pulled out of the pellicle. When it is broken off, the break occurs at the pellicle. In some cases (Fig. 2) the cilium is broken on both sides of the pellicle. Nevertheless, the ciliary ring remains plugged with a cross sectional segment of cilium.

#### *The kinetosome*

In the opinion of the writers, the bulb or knob at the internal terminus of the cilium is the ciliary basal body or kinetosome. This structure has a diameter (*P. multimicronucleatum*) of approximately 0.27$\mu$ (the kinetosome is assumed to have suffered no appreciable flattening since it casts a respectable shadow). This value is about 2.5 times that obtained for the cilium base and the fibril base (0.1 and 0.12$\mu$ in diameter, respectively; calculated on the assumption that these structures are flattened cylinders in the photomicrographs). Comparable values were obtained in *P. aurelia*. When two ciliary rings occur in one polygon, two corre-

FIGURE 7. *Paramecium aurelia*. A segment of the two middle kineties of Figure 6. Approximately 60,800 ×.

FIGURE 8. *P. multimicronucleatum*. A single kinetodesmal bundle extending beyond the torn pellicle. Terminal portions of five component fibrils are shown. These fibrils are seen to overlap in shingle-like fashion. Approximately 15,400 ×.

sponding kinetosomes appear. This is most evident in the oral region (Fig. 3).

In some figures (*i.e.*, 6, 7) the kinetosome seems to be grooved in the direction of the cilium. Otherwise there is no indication of internal structure or multiple organization. No accessory kinetosomes to correspond to Nebenkörner (Klein, 1928; von Gelei, 1932) occur in any of the figures.

### *The kinetodesma*

Studies with the light microscope show that the kinetosomes are connected by a longitudinal fiber, the kinetodesma or neuromotor fiber. The electron microscope resolves this into a bundle of fibrils (especially clear in Figs. 6 and 7). Each of the component fibrils in the kinetodesmal bundle arises independently from a kinetosome, passes to one side and joins the main bundle. The kinetodesmal fibrils are not of indefinite length and constant diameter, but taper gradually to end in the main bundle. Thus, the main kinetodesmal bundle consists of a number of fibrils that overlap in shingle-like fashion along the bundle. This is most clearly seen in Figure 8. Here a bundle extends beyond the torn edge of the pellicle. The terminal portions of five component fibrils are clearly shown in this figure. The period between the ends of adjacent fibrils averages approximately 2.3$\mu$. This value compares favorably with the interkinetosome interval (2.0 $\mu$) calculated from another specimen. Evidently then, the kinetodesma of the light microscopist actually consists of a number of fibrils. Each of these arises independently from a kinetosome, joins the bundle and gradually tapers to a fine point. Apparently the kinetodesmal fibrils are of constant length, at least in a limited segment of the bundle. When two ciliary rings with the corresponding kinetosomes occur in a single polygon, both kinetosomes are joined to a single kinetodesmal fibril. This is particularly evident in the oral area.

To judge from the position of the trichocyst attachment points in the outer lattice and the arrangement of the polygons in the pellicular fragments, the kinetodesmal bundles must assume an approximately longitudinal course, with respect to the axis of the animal, except in the oral region. This agrees with the findings of earlier workers (*i.e.*, Lieberman, 1929). Not only do the bundles run longitudinally but the component fibrils are definitely polarized with respect to the axis of the animal. All of the fibrils join the bundle and pass along it in one direction. This is clearly seen in Figures 2, 4 and 6 and is true of every specimen examined that did not clearly represent part of the oral region. In the absence of any anterior or posterior reference point in the fragments it is not immediately clear whether the fibrils run from a posterior to a more anterior region or the reverse. But assuming that the "rule of desmodexy" (Lwoff, 1950) applies in Paramecium, it can be stated with some degree of confidence that the fibrils proceed in an anterior direction from the kinetosomes. According to the rule of desmodexy the kinetosomes lie to the right of the corresponding kinetodesma as the ciliate is viewed in normal orientation. Inspection of the figures shows that the kinetodesmal bundles lie to one side of the kinetosomes and that the individual fibrils curve laterally from their kinetosomal origins to join the main bundle. This condition is probably exaggerated somewhat by the action of surface tension when the preparation is dried. In this connection it should be noted that the kinetodesma appears as a line directly connecting adjacent kinetosomes in most light microscope studies.

In any event, the kinetodesma would necessarily have to deviate sufficiently from a straight course to pass around the trichocyst. Most of the figures presented here are so oriented that the kinetosomes lie to the right of the main kinetodesmal bundle as the fragment is viewed from its outer surface in accordance with desmodexy. In some of the figures (2, 3, 6 and 7) the pellicle is viewed from inside the animal. In these the kinetosomes will appear to the left of the kinetodesmal bundle. When oriented in this way it is apparent that the fibrils pass upward. Thus, if desmodexy applies in Paramecium, it is evident that the fibrils pass in an anterior direction from the kinetosomes.

The arrangement of the circumoral kinetodesmas adds support to this view. At least in the *aurelia-caudatum-multimicronucleatum* group of paramecia the kinetodesmas on the left ventral side of the animal (as viewed from the dorsal surface) pass forward, curve around the anterior margin of the oral opening and finally terminate in the preoral suture (Lieberman, 1929). The kinetodesmas on the right ventral side remain more nearly parallel to the suture. Therefore, the system in the oral region is asymmetrical and an isolated oral fragment can be oriented with respect to its position in the animals. When this is done (Fig. 3) all the kinetodesmal fibrils, on both the left and the right sides of the oral opening, are seen to pass in an anterior direction from their kinetosomes.

### *Structure of the individual kinetodesmal fibrils*

No special effort was made in this study to investigate the structure of the individual kinetodesmal fibrils. Nevertheless, certain interesting features are evident in the preparations. These tapering fibrils show a periodic structure when shadowed longitudinally. In the best figures (*P. calkinsi,* Fig. 5) this takes the form of a spiral with a calculated period of approximately 400 Å. The spiral organization extends from the base to the tip of the fibril and its period appears to remain constant throughout the length of the fibril in spite of the fact that the fibril tapers to a point from a base diameter of approximately 0.12 $\mu$ (*P. multimicronucleatum*). It should be noted that the electron photomicrographs reveal this structure as a spiral ridge in the surface of the fibril. This ridge resembles the threads of a screw and casts the shadows which resolve the spiral in the photomicrographs. In order to preserve definition Figure 5 was not "reversed." Therefore, shadows appear light against a dark background in this figure.

The fibril never frays or breaks down in the preparations. It usually remains intact throughout its length in *P. aurelia, P. caudatum, P. multimicronucleatum,* but breaks off rather frequently in *P. calkinsi.* The fibrils themselves appear to be the most resistant parts of the system. They sometimes break free at their bases from their kinetosomes (Fig. 4) or the kinetosomes and the proximal stumps of the cilia may disintegrate (Fig. 2), but the fibrils remain intact and retain their characteristic organization. A thorough study of these extraordinary structures should prove very interesting. Perhaps more details could be resolved by partial digestion with enzymes or by examining sections at high magnification.

It is not clear how the overlapping fibrils are held together to form the kinetodesmal bundles. In some specimens the individual fibrils appear to be tightly bound together (Figs. 1, 3), but in others the fibrils are separated to a greater or less degree (Figs. 2, 6, 7, 8). Occasionally they even appear as completely isolated structures. Presumably the tightly bound condition is the natural one. The binding agency must involve strong forces, for in many of the preparations (see Fig. 4) a number of fibrils are free from their kinetosomal anchors but nevertheless remain bound to the bundle in normal orientation. No evidence for a sheath about the bundle or any appreciable cementing material between individual fibrils has been found to account for this.

### *Commissural connectives*

Some previous investigators have described cross connecting or commissural fibers extending laterally to adjacent kinetodesma. Lund (1933) figures these as connecting kinetosomes and states that they occur occasionally in the general body fibrillar system and that they form a complex lattice in the oral region. In the present study no evidence for such commissural connectives was found outside the oral area. Here, however, individual kinetodesmal fibrils sometimes pass at right angles to one another (Fig. 3). These may represent the commissural connectives of Lund.

## Discussion

Studies on protozoan fibrillar systems are pertinent to several fields of interest. They contribute to the knowledge of protozoan morphology; they apply to the physiological problems of conduction, coordination and contraction; and they concern problems of morphogenesis. These aspects of the present investigation are considered below.

### *Morphological considerations*

The main features of the pellicular lattice and kinetodesmal system of Paramecium, as seen in this study, agree with the findings of most previous workers. Indeed they confirm the views of J. von Gelei (1925), Lund (1933) and Worley (1933) in a most striking manner. The outer lattice is quite evidently distinct and anatomically separate from the primary or inner system. The only possible exception to this could be a connection between the trichocyst attachment point and the cilium ring in the pellicle. No evidence for any direct connection between the trichocysts and the kinetodesmal system was found. The study does not support Klein's (1926) concept of an indirect connection between the lattice and kinetodesmal system.

Certain other fibrillar structures have been described in Paramecium. Rees (1922) reported a system in which fibers arise from the kinetosomes, pass into the endoplasm and then proceed more or less directly to a neuromotorium located in the vicinity of the cytopharynx. No suggestion of the fibers of Rees was found in the electron photomicrographs. G. von Gelei (1937) has described still another fiber system. This constitutes a lattice at the level of the kinetosomes which ramifies throughout the animal at this level. No evidence for this system appeared in the current investigation. Although this study does not confirm the existence of these structures, it cannot be considered as critical evidence for their absence in view of the method of preparation of the material (see section on methods).

A number of investigators have described the kinetosome region of Paramecium as a compound structure (Klein, 1928; J. von Gelei, 1932; Sedar, 1952) containing two or more distinct granules. One of these is usually considered to be a true ciliary basal body; the remainder as accessory bodies or Nebenkörner. In three of the four species examined with the electron microscope the kinetosomes appear to be single structures. In the exceptional form, *P. calkinsi,* two bodies which may be kinetosomes are frequently associated with each pellicular polygon. The study of this form is not sufficiently complete to determine whether this condition is general over the body surface or whether it applies only to the oral region as in *P. aurelia.* Likewise the detailed relationship of these bodies to the kinetodesmal fibrils in *P. calkinsi* has not yet been worked out.

Possibly compound kinetosomes may yet be found in *P. aurelia, P. multimicronucleatum* and *P. caudatum* after sonic treatment if the fixation procedure is varied. But whether or not such accessory structures are found, there is no question that at least one such structure joins the base of the cilium to the kinetodesmal fibril and that bundles of these tapering fibrils overlap to form the kinetodesma of the light microscopist.

Concerning this relationship of kinetosome to the cilium and kinetodesma, Worley's (1933) Figure 2 is of interest. This figure, a photomicrograph of a fresh preparation, clearly shows a series of bodies from which short fibers arise. These fibers appear to overlap. Worley makes no comment concerning this overlapping condition although he does state that direct connecting fibers (kinetodesma) are visible in the figure. However, it appears likely that these fibers are cilia which all lie in one direction.

### *Physiology*

Nearly every student of Paramecium fibrillar systems has attributed specific functions to the various fibrillar structures. These functions include physical support, contraction, specific metabolic activity and various aspects of ciliary coordination. Unfortunately, few investigators have made any experimental attempt to support their theses, but rather follow the procedure of Lund (1933) who, on the basis of morphology alone, concludes (p. 58) that a correlating function "is almost to be accepted without choice" in the case of the kinetodesma. The most serious attempt to investigate the function of fibrillar systems in Paramecium by experimental means appears to be the study of Worley (1934).

Employing microdissection methods, this investigator made short transverse cuts through the ectoplasm of Paramecium and noted that the metachronal waves of ciliary action did not proceed beyond the cut. Metachronism was not disturbed elsewhere. Evidently, control of metachronism resides in the ectoplasm and the control follows longitudinal paths. In Worley's experiments ciliary reversal occurred simultaneously over the entire body surface and on both sides of ectoplasmic cuts. This suggests that the kinetodesmal fibrils do not control ciliary reversal. Although this evidence is neither extensive nor overwhelming, it does support the view that the kinetodesmal system has a role in ciliary coordination. For more extensive treatment of this aspect of the subject the reader should consult Parker (1929), Taylor (1941), Prosser *et al.* (1950) and Wichterman (1953).

The present demonstration that the kinetodesmas are not single fibers but consist of bundles of relatively short overlapping fibrils, each connected independently to a kinetosome, introduces a complication in any coordination theory of kinetodesmal action. Coordination would have to pass laterally from one fibril to another— presumably to the fibril of the next adjacent cilium. This would imply a high degree of orientation of the fibrils with respect to each other and also that the relationship has the properties of a lateral synapse. In this connection it is of interest that *Tetrahymena* contains a specific acetylcholine esterase (Seaman and Houlihan, 1951) and that this enzyme appears to be confined to the ectoplasm of the ciliate (Seaman, 1951).

Many ciliates are highly contractile organisms. Although Paramecium is not notable in this regard, some investigators (Brown, 1930) have ascribed a contractile function to certain of the fibrillar elements. If Paramecium showed clear-cut contractile properties, it would be tempting to ascribe such action to the spiral organization of the kinetodesmal fibrils.

### *Morphogenesis*

In ciliates the kinetosomes appear to be self-reproducing, genetically continuous entities; in certain forms they may act as pluripotent organization centers and finally they may be the seat of morphogenetic dominance and polarity (Lwoff, 1950; Weisz, 1951).

In Paramecium the demonstrated behavior and properties of the kinetosomes are somewhat less spectacular. During fission or as a preliminary to fission all body kinetosomes divide one or more times, the daughter granules separate along the longitudinal axis of the animal and finally form new cilia (von Gelei, 1934b; Downing, 1951). The kinetosomes of the oral region may be responsible for the specialized oral morphology and ciliature, since the new oral region is formed by a budding process from the original at fission (Hertwig, 1889; von Gelei, 1934b). The morphogenetic agents in the bud could be kinetosomes although evidence for this view is lacking. The origin of trichocysts is obscure (Wichterman, 1953); von Gelei (1934b) believes that new trichocyst granules arise from the neuronemes (kinetodesmas), a view which agrees roughly with that of Lwoff (1950) for apostomatous ciliates.

Although the present study was not designed specifically to clarify morphogenetic problems, it does re-define certain of these at a finer morphological level. This applies particularly to the problems of duplication and polarity of kineties. An individual kinety is seen to be a compound structure composed of definite units. Each unit is composed of a cilium, a kinetosome and a short, tapering kinetodesmal fibril. How, then, do these units duplicate and orient to form the compound structure of the kinety? According to current views the kinetosome is the primary duplicating organelle. By unknown mechanisms it can reproduce itself and it can produce other organelles. In keeping with this concept an individual kinetosome of Paramecium should be able to reproduce itself and produce a cilium with its complex internal structure. Ninety degrees from the site of cilium origin it should produce a kinetodesmal fibril with its taper and spiral symmetry. In the course of this study a single forked kinetodesmal fibril with two tapering ends was encountered. This unusual fibril could have arisen as a "developmental anomaly" from a kinetosome, but it is also possible that duplication is regularly initiated at the tip of the fibril and proceeds proximally to include the kinetosome.

In all of the preparations examined the kinotodesmal fibrils are polarized. They all taper in one direction and form tight bundles. If the kinetodesmal fibrils develop from the kinetosomes, "contact guidance forces" (Weiss, 1945) of some sort might cause a developing fibril to proceed along a pre-existing bundle, but such a concept does not so readily explain the exclusive anterior orientation in this polarity.

Refinement of the methods used here and their application to a variety of forms in stages of division and regeneration, in conjunction with sectioning techniques, may be expected to provide some insight into these problems.

The writers wish to express their great indebtedness to the late Professor Harold Kirby. With characteristic generosity Professor Kirby made his personal laboratory available to us for an investigation of Paramecium. It is a source of the deepest regret that Professor Kirby did not live to see the present study emerge.

We wish to express our appreciation to Dr. E. W. Steinhaus, Dr. K. M. Hughes and Dr. A. H. Gold of the University of California for technical aid. The electron microscope at North Carolina State College was used in the later phases of the study. We wish to thank Dr. A. C. Menius and Mr. W. Withers for the use of this instrument and for their assistance.

## SUMMARY

1. Various structures in Paramecium are readily obtained for electron microscope examination by formalin fixation followed by sonic dissection. These structures include macronuclei, mitochondria, trichocysts in various stages of explosion and fragments of the pellicle. This study concerns such fragments.

2. The electron microscope reveals two fiber systems associated with the pellicular fragments. One of these is intimately associated with the pellicular membrane and corresponds to the outer fibrillar lattice system of earlier investigators. The second system is subpellicular and corresponds to the kinety, neuroneme, or inner fiber system.

3. The pellicle proper consists of a thin membrane with no obvious fine structure. The outer lattice system is a continuous network. It corresponds in position to the system of polygonal ridges in the pellicle. A thickening in the cross bars of the polygonal lattice represents the trichocyst attachment point. A ring-shaped structure is found in the center of each polygon. The cilium passes through this ring. A short distance from this ciliary ring a second, smaller ring-shaped thickening is frequently found.

4. The kineties are compound structures composed of discrete units. Each unit consists of three parts: a) the cilium which passes through the ciliary ring of the pellicle and terminates internally at b), the ciliary basal body or kinetosome. Each kinetosome gives origin to c), a tapering fibril which parallels the surface of the animal. These units are associated by their tapering fibrils. The fibrils from a longitudinal row of kinetosomes overlap in shingle-like fashion to form a tight bundle. This bundle is the kinetodesma of the light microscopist. The fibrils of the kinetodesma all taper toward the anterior end of the animal.

5. No obvious connection exists between the outer lattice system and the kinety system.

## LITERATURE CITED

Beams, H. W., and R. L. King, 1941. Some physical properties of the protoplasm of the protozoa. *In:* Calkins, G. N., and F. M. Summers, Protozoa in Biological Research. Columbia University Press, New York.

Brown, V. E., 1930. The neuromotor apparatus of *Paramecium*. *Arch. Zool. Exp. Gen.*, **70**: 469–481.

Downing, W. L., 1951. Structure and morphogenesis of the cilia and the feeding apparatus in *Paramecium aurelia*. *Proc. Amer. Soc. Protozool.*, **2**: 14–15.

Fauré-Fremiet, E., 1948. Les mécanismes de la morphogenèse chez les ciliés. *Folia Biotheoretica*, **111**: 25–58.

Finley, H. E., 1951. Electron microscopy of thin-sectioned *Spirostomum*. *Science*, **113**: 362–363.

von Gelei, G., 1937. Ein neues Fibrillensystem im Ectoplasma von *Paramecium*. *Arch. f. Protistenk.*, **89**: 133–162.

von Gelei, J., 1925. Ein neues Paramaecium aus der Umgebung von Szeged. *Paramaecium nephridiatum* n. sp. *Allatt. Közlem.*, **22**: 121–159. (Résumé in German, 245–248.)

von Gelei, J., 1932. Die reizleitenden Elemente der Ciliaten in nass hergestellten Silber-bzw. Goldpräparaten. *Arch. f. Protistenk.*, **77**: 152–174.

von Gelei, J., 1934a. Der feinere Bau des Cytopharynx von *Paramecium* und seine systematische Bedeutung. *Arch. f. Protistenk.*, **82**: 331–362.

von Gelei, J., 1934b. Das Verhalten der ectoplasmatischen Elemente des Parameciums während der Teilung. *Zool. Anz.*, **107**: 161–177.

Hertwig, R., 1889. Ueber die Conjugation der Infusorien. *Abhandl. bayer. Akad. Wiss.*, **17**: 150–233.

Jakus, M. A., and C. E. Hall, 1946. Electron microscope observations of the trichocysts and cilia of *Paramecium*. *Biol. Bull.*, **91**: 141–144.

Klein, B. M., 1926. Ergebnisse mit einer Silbermethode bei Ciliaten. *Arch. f. Protistenk.*, **56**: 243–279.

Klein, B. M., 1928. Die Silberliniensysteme der Ciliaten. Weitere Resultate. *Arch. f. Protistenk.*, **62**: 177–260.

Lieberman, P. R., 1929. Ciliary arrangement in different species of *Paramecium*. *Trans. Amer. Microscop. Soc.*, **48**: 1–11.

Lund, E. E., 1933. A correlation of the silverline and neuromotor systems of *Paramecium*. *Univ. Cal. Publ. Zool.*, **39**: 35–76.

Lwoff, A., 1950. Problems of morphogenesis in ciliates. John Wiley and Sons, New York.

Metz, C. B., 1947. Induction of "pseudo selfing" and meiosis in *Paramecium aurelia* by formalin killed animals of opposite mating type. *J. Exp. Zool.*, **105**: 115–140.

Metz, C. B., 1953. Mating substances and the physiology of fertilization in ciliates. Sex in Microorganisms. *A. A. A. S. Symposium Volume*. In press.

Parker, G. H., 1929. The neurofibril hypothesis. *Quart. Rev. Biol.*, **4**: 155–178.

Pitelka, D. R., 1949. Observations of flagellum structure in Flagellata. *Univ. Cal. Publ. Zool.*, **53**: 377–430.

Pitelka, D. R., and C. B. Metz, 1952. The structure of the "neuromotor apparatus" of *Paramecium* as revealed by the electron microscope. *Biol. Bull.*, **103**: 282–283.

Prosser, C. L., F. A. Brown, Jr. et al., 1950. Comparative animal physiology. W. B. Saunders Company, Philadelphia.

Rees, C. W., 1922. The neuromotor apparatus of *Paramecium*. *Univ. Cal. Publ. Zool.*, **20**: 333–364,

Seaman, G. R., 1951. Localization of acetylcholinesterase activity in the protozoan, *Tetrahymena geleii S. Proc. Soc. Exp. Biol. Med.*, **76**: 169–170.

Seaman, G. R., and R. K. Houlihan, 1951. Enzyme systems in *Tetrahymena geleii* (S). II. Acetylcholinesterase activity. Its relation to motility of the organism and to coordinated ciliary action in general. *J. Cell. Comp. Physiol.*, **37**: 309–322.

Sedar, A. W., 1952. Electron microscope studies on the structure and morphogenesis of the cilia and silverline system of *Paramecium multimicronucleatum*. *Proc. Amer. Soc. Protozool.*, **3**: 12–13.

Sonneborn, T. M., 1950. Methods in the general biology and genetics of *Paramecium aurelia. J. Exp. Zool.,* **113**: 87–147.

Tartar, V., 1941. Intracellular patterns: facts and principles concerning patterns exhibited in the morphogenesis and regeneration of ciliate Protozoa. *Third Growth Symposium, 21–40.*

Taylor, C. V., 1941. Fibrillar systems in ciliates. *In:* Calkins, G. N., and F. M. Summers, Protozoa in Biological Research. Columbia University Press, New York.

Weiss, P., 1945. Experiments on cell and axon orientation in vitro: the role of colloidal exudates in tissue organization. *J. Exp. Zool.,* **100**: 353–386.

Weisz, P. B., 1951. A general mechanism of differentiation based on morphogenetic studies in ciliates. *Amer. Nat.,* **85**: 293–311.

Wichterman, R., 1953. The biology of *Paramecium.* The Blakiston Company, Inc., New York.

Worley, L. G., 1933. The intracellular fibre systems of *Paramecium. Proc. Nat. Acad. Sci. Wash.,* **19**: 323–326.

Worley, L. G., 1934. Ciliary metachronism and reversal in *Paramecium, Spirostomum* and *Stentor. J. Cell. Comp. Physiol.,* **5**: 53–72.

# THE MECHANISM OF SYNERGISTIC ACTION OF DMC WITH DDT AGAINST RESISTANT HOUSE FLIES

ALBERT S. PERRY, ARNOLD M. MATTSON AND ANNETTE J. BUCKNER

*U. S. Public Health Service, Federal Security Agency, Communicable Disease Center, Savannah, Georgia*

With the recent widespread occurrence of house fly resistance to many of the halogenated hydrocarbons, intensive efforts have been made to find chemicals which might enhance the effectiveness of these insecticides, especially DDT, against resistant house flies.

In the course of searching for such materials, Perry and Hoskins (1950, 1951a) found that piperonyl cyclonene increased markedly the effectiveness of DDT against various strains of DDT-resistant house flies. Sumerford, Goette, Quarterman and Schenck (1951) reported on the potentiation of DDT against resistant house flies by several structurally related compounds including 1,1-bis-(*p*-chlorophenyl) methyl carbinol (DMC). In a later work, Sumerford, Fay, Goette and Allred (1951) discussed the results of preliminary screening of several other candidate synergists for DDT. More recently, March, Metcalf and Lewallen (1952) have added to this list several effective synergists for DDT and closely related compounds.

Although a substantial amount of data has been reported on the potentiation of various insecticides by so-called synergists, little attention has been given to the biochemical significance of this phenomenon.

Various explanations have been advanced regarding the mode of action of synergistic compounds. David and Bracey (1944) attributed the action of several synergists in pyrethrum fly sprays to a delay in the knockdown of test insects, resulting in longer flying periods and in accumulation of larger amounts of the toxicant. Parkin and Green (1944), on the other hand, showed that retardation of knockdown was not a factor in synergistic action. Dove (1947) concluded that pyrethrum residues are stabilized in thin films by certain synergists, particularly piperonyl butoxide. Munro (1942), Lindquist, Madden and Wilson (1947) and others suggested that pyrethrum synergists may assist the penetration of the toxicant through the insect cuticle. Page and Blackith (1949) proposed that a loose molecular complex is formed between pyrethrins and synergists, and that the presence of those synergists influences the orientation of the pyrethrin molecules at the nerve-sheath interface, resulting in a greater concentration of the toxicant at the site of action. The observations made by Wilson (1949) on the action of piperonyl butoxide and piperonyl cyclonene with pyrethrum on house flies led him to conclude that the synergists damage a detoxifying mechanism. The detailed studies of Chamberlain (1950) on the mode of action of piperonyl butoxide with pyrethrins indicated that the enzyme lipase, obtained from roach and house fly extracts, was, in part, responsible for the detoxification of pyrethrins, and that inclusion of piperonyl butoxide in the formulation produced some degree of inhibition of the hydrolytic action of lipase. More recently, Perry and Hoskins (1950, 1951b) demonstrated that the synergistic action with DDT imparted by piperonyl cyclonene was not the result of increased permeability of the house fly cuticle but was largely due to inhibition of detoxification of DDT.

The purpose of this report is to give a detailed account of the effect of 1,1-bis-(*p*-chlorophenyl) methyl carbinol (DMC) as a synergist for DDT, and to show a quantitative relationship between synergistic action and inhibition of DDT detoxification in resistant house flies.

## Materials and Methods

The following materials were used in this study:

*p,p'*DDT; 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane, recrystallized twice from ethanol; m.p. 108° C.

*p,p'*DDE; 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene, obtained by dehydrochlorination of DDT in alcoholic KOH solution; m.p. 88° C.

*p,p'*DMC; 1,1-bis-(*p*-chlorophenyl) methyl carbinol, prepared from the technical product by fractional distillation under vacuum, followed by recrystallization from petroleum ether; m.p. 67–68° C. (*cf.* Grummitt, Buck and Becker, 1945).

*p,p'*DME; 1,1-bis-(*p*-chlorophenyl) ethylene, prepared by dehydration of DMC in the presence of anhydrous copper sulfate. The crude product obtained was purified by vacuum distillation, followed by recrystallization from petroleum ether and from ethanol; m.p. 84° C. (*cf.* Grummitt, Buck and Becker, 1945).

Application of benzene solutions of the chemicals was made topically by means of a micro-loop. The calibrated [1] micro-loop gave an average volume delivery of 0.00065 ml. (0.65 mm.$^3$) with less than 5 per cent error. The desired dosages were obtained by proper dilution of the solutions. The strain of flies used in this work was a multiple-resistant strain obtained from a local dairy and designated as Roberds. In a typical experiment, groups of 50 to 100 adult female flies, three to four days old, were anesthetized with carbon dioxide and the desired dosage was applied topically to the ventral thoracic region of individual flies. Following exposure, the flies were placed in holding cages provided with food and water and were kept at 26° C. and 60–70 per cent relative humidity. Mortality counts were made 24 hours after exposure.

The procedure used for studying the degradation of DDT and DMC was as follows: At a chosen interval after exposure, the flies were thoroughly rinsed in three successive 20-ml. portions of n-hexane to remove adhering particles of the chemicals. They were then ground in the presence of anhydrous sodium sulfate and extracted with carbon tetrachloride by mechanical agitation for one hour, and then filtered off. The external and internal extractions were tested for DDT and DDT-metabolites by the procedure of Schechter, Soloway, Hayes and Haller (1945). The latter procedure was also used in the determination of DMC and related compounds. The method of computation for mixtures of DDT and DDE was described in an earlier paper (Perry and Hoskins, 1951b). Since the Schechter-Haller color complexes of DDE, DMC and the ethylene derivative of

---

[1] Data from the Communicable Disease Center, Technical Development Branch, Public Health Service, Summary of Activities No. 25, p. 170, 1951.

DMC, 1,1-bis-(*p*-chlorophenyl) ethylene (designated as DME for convenience) have very similar absorption spectra, it is necessary to separate these compounds before quantitative determinations of each in a mixed solution can be made. To accomplish the separation of DMC and DME from DDT and DDE, use was made of a sulfuric acid-Celite column similar to that described by Davidow (1950) for the isolation of DDT from fat. A column (2.5 × 25 cm.) fitted with a glass stopcock was packed to a depth of 10 cm. The various materials dissolved in carbon tetrachloride were added in 100-microgram quantities to the columns and eluted with carbon tetrachloride until 100 ml. of eluate had been collected. It was found that DMC and DME, when chromatographed singly or in combination with DDT and DDE, were retained by the column (Table I). However, no satisfactory method was found for recovering quantitatively the DMC and DME retained by the acid-Celite mixture.

TABLE I

*Separation of DDT and DDE from DMC and DME by sulfuric acid-Celite column*

| Number | Materials partitioned (100 microgram quantities) | Per cent recovery in eluate | | | |
|---|---|---|---|---|---|
| | | DDT | DDE | DMC | DME |
| 1 | DDT | 93.9 | — | — | — |
| 2 | DDE | — | 102.6 | — | — |
| 3 | DMC | — | — | None | — |
| 4 | DME | — | — | — | None |
| 5 | DDT<br>DMC | 85.4 | — | None | — |
| 6 | DDT<br>DME | 95.6 | — | — | None |
| 7 | DDE<br>DMC | — | 96.3 | None | — |
| 8 | DDT<br>DMC<br>DDE | 96.5 | 96.1 | None | — |
| 9 | DDT<br>DME<br>DDE | 94.3 | 99.8 | — | None |
| 10 | DDT<br>DMC<br>DME | 87.1 | — | None | None |

In separating the various materials from fly tissues the extracts were evaporated, redissolved in 20 ml. of carbon tetrachloride and filtered through the column.

## RESULTS

The range of mortalities of the Roberds strain resulting from topical applications of benzene solutions of *p,p'*DDT alone or in combination with *p,p'*DMC are shown in Table II. For convenience in comparing the effectiveness of the many combinations used, the data are plotted in Figure 1 as log-dosage of DDT in micrograms per fly versus mortality on the probit scale. The dosages of DMC in micrograms per fly are given on each regression line. These data permit determination of the amount of DDT required for any level of mortality with a given dosage of the synergist.

The synergistic effect resulting from separate applications of DDT and DMC was determined by pre-treating resistant flies with DMC at 6- and 24-hour in-

TABLE II

*Twenty-four-hour mortality (per cent) of adult female house flies (Roberds strain) resulting from topical application of DDT and DMC at various ratios. Control tests showed no mortality*

| Micrograms/fly | | Per cent mortality | |
|---|---|---|---|
| DDT | DMC | Range | Average |
| 0.32 | 0.65 | 2–14 | 8 |
| | 3.25 | 10–25 | 17 |
| | 6.50 | 20–30 | 25 |
| 0.65 | 0.06 | 2–20 | 12 |
| | 0.32 | 15–50 | 31 |
| | 0.65 | 40–72 | 53 |
| | 3.25 | 72–98 | 85 |
| | 6.50 | 92–98 | 95 |
| 1.62 | 0.06 | 10–22 | 17 |
| | 0.32 | 44–88 | 54 |
| | 0.65 | 78–98 | 87 |
| | 3.25 | 100 | 100 |
| 3.25 | 0.06 | 26–68 | 41 |
| | 0.32 | 68–96 | 86 |
| | 0.65 | 100 | 100 |
| 6.50 | 0.06 | 42–58 | 50 |
| | 0.32 | 90–96 | 93 |
| | 0.65 | 100 | 100 |
| 13.0 | 0.06 | 61–75 | 68 |
| 6.5 | — | 8–30 | 15 |
| 13.0 | — | 24–62 | 40 |
| 26.0 | — | 46–75 | 67 |
| 52.0 | — | 72–98 | 88 |
| — | 6.50 | 0 | 0 |
| — | 13.0 | 0 | 0 |

tervals before treatment with DDT. The reverse procedure, *i.e.*, application of DDT followed by DMC at the same intervals was also used. Simultaneous applications of DDT and synergist were used for comparison. In each case mortality counts were made 24 hours after application of the second compound. The results (Table III) indicate a marked decrease in mortality when applications of DDT and DMC were separated by 6 and 24 hours, irrespective of the order of application and as long as the dosage of DDT was kept low. With the higher dosages of DDT it is evident that application of DDT followed by DMC at the indicated intervals was more effective than the reverse procedure. This may be explained on the assumption that sufficient unchanged DDT was still present when the DMC was applied, especially at the 6-hour interval, to produce high synergistic activity. Since in all cases simultaneous applications of DDT and DMC showed greater activity than separate applications of the chemicals, an explanation of the cause of synergistic action was sought in changes undergone by DMC after absorption.

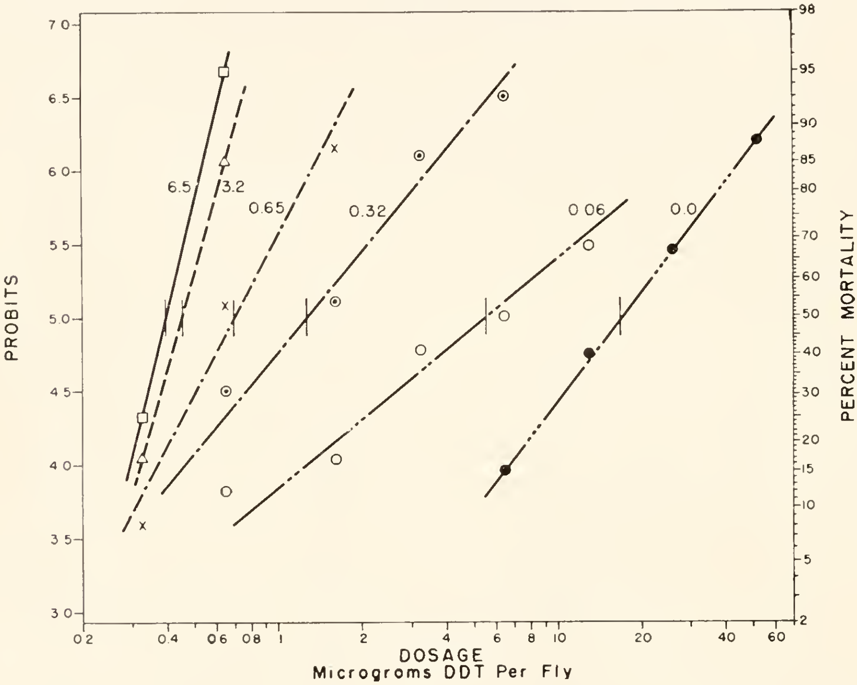


FIGURE 1. Probit-log dosage lines for adult female house flies (Roberds strain); DMC in micrograms per fly as indicated on the curves, plus various amounts of DDT applied topically to the ventral thoracic region.

To test this hypothesis, groups of flies were given topical applications of DMC in the usual manner. At various intervals ranging from 2 to 72 hours after exposure the flies were sacrificed and analyses of external and internal DMC were made by the procedure described earlier. Table IV shows that 24 hours after application of 13.0 micrograms DMC per fly, external rinsing removed approximately

Table III

*Dosage-mortality data for simultaneous and separate applications of DDT and DMC at various intervals. DDT at 6.50 micrograms per fly gave an average mortality of 12 per cent. DMC had no effect at highest dosage shown*

| Material and dosage in micrograms/fly | | Per cent mortality | | | | |
|---|---|---|---|---|---|---|
| | | | DDT followed by DMC | | DMC followed by DDT | |
| DDT | DMC | DDT:DMC mixture | Hours | | | |
| | | | 6 | 24 | 6 | 24 |
| 0.32 | 3.25 | 26 | 0 | 0 | 12 | 2 |
| 0.65 | 0.65 | 48 | 0 | 0 | 6 | 2 |
| 0.65 | 3.25 | 82 | 8 | 0 | 60 | 4 |
| 3.25 | 0.32 | 89 | 80 | 16 | 24 | 0 |
| 6.50 | 0.32 | 96 | 90 | 58 | 64 | 10 |

0.4 micrograms DMC and internal extraction yielded 1.1 micrograms, giving a total of 1.5 micrograms, equivalent to 11.5 per cent recovery. Hence the undetected material, equivalent to 88.5 per cent, was either excreted or changed to some other product which did not respond to the method of analysis.

The former assumption was tested by collecting the excreta from DMC-treated flies held with food and water in covered beakers. At the end of the holding period, usually 24 hours, the flies were sacrificed and analyzed in the usual manner. The excreta from the beakers, food containers and cheesecloth covers were thoroughly washed with several portions of 2 per cent aqueous solution of sodium hydroxide followed by several rinses with n-hexane. The solvent and alkali were combined and shaken for several minutes, after which they were centrifuged and the layers separated. Analysis of the hexane portion showed the presence of only small amounts of DMC (Table V). The alkali layer was therefore acidified to pH 2–3 with 6 N sulfuric acid and extracted with ether to determine if any

Table IV

*Amounts of external and internal DMC recovered from adult female house flies (Roberds strain) at various intervals following topical application of 13.0 micrograms DMC per fly*

| Interval to extraction (hours) | DMC recovered | | | | Per cent DMC unaccounted (by difference) |
|---|---|---|---|---|---|
| | External | | Internal | | |
| | µg./fly | Per cent | µg./fly | Per cent | |
| 2 | 7.65 | 58.8 | 4.50 | 34.6 | 6.6 |
| 4 | 5.75 | 44.2 | 6.50 | 50.0 | 5.8 |
| 8 | 2.05 | 15.7 | 4.46 | 34.3 | 50.0 |
| 16 | 0.65 | 5.0 | 1.80 | 13.8 | 81.2 |
| 24 | 0.55 | 4.2 | 1.48 | 11.4 | 84.4 |
| 48 | 0.25 | 2.0 | 0.54 | 4.1 | 93.9 |
| 72 | 0.17 | 1.3 | 0.50 | 3.8 | 94.9 |

acidic derivatives of DMC might be found. Results of four to six tests, which were averaged for each dosage (Table V), indicated the presence in the excreta of a metabolic product of DMC tentatively identified as bis-(*p*-chlorophenyl) acetic acid (DDA), on the basis of its solubility in dilute alkali. Orienting tests showed that DMC and DME are not removed by dilute alkali. None of the above products was found in extracts from untreated flies which served as controls.

From chemical considerations it appears that the conversion of DMC to DDA must proceed through the mediation of some intermediate product(s). The ease with which DMC undergoes dehydration *in vitro* suggests that the first product of degradation might be the ethylene derivative, 1,1-bis-(*p*-chlorophenyl) ethylene (DME). Tests with DME, similar to those described above for DMC, also showed the presence in the excreta of the same product, DDA (Table V). Topical applications to resistant flies of DDT-DME combinations evinced some synergistic activity, although not as marked as that with DDT-DMC. This might also serve as an indication of the intermediate role played by DME in the metabolism of DMC. In addition to DME, it is conceivable that one or possibly more intermediate(s) are formed in the conversion of DMC to DDA.

TABLE V

*Determination of DMC, DME and their degradation products 24 hours following topical application of DMC or DME to adult female house flies. Average of four to six tests with each dosage*

| Material and dosage applied in micrograms per fly | Product recovered | From tissue extracts | | From excreta | |
|---|---|---|---|---|---|
| | | Micrograms per fly | % of amount applied | Micrograms per fly | % of amount applied |
| DMC 6.5 | DMC and/or DME | 0.69 | 10.7 | 0.32 | 4.8 |
| | DDA | 0.62 | 9.5 | 4.20 | 64.6 |
| DMC 13.0 | DMC and/or DME | 1.62 | 12.5 | 0.66 | 5.0 |
| | DDA | 0.84 | 6.4 | 6.80 | 52.3 |
| DME 13.0 | DME | 1.30 | 10.0 | 0.18 | 1.4 |
| | DDA | 0.70 | 5.4 | 6.50 | 50.0 |

The degradation of DDT to the relatively nontoxic derivative DDE has been shown by Sternburg, Kearns and Bruce (1950). Perry and Hoskins (1950, 1951b) and Fullmer and Hoskins (1951) further demonstrated the inhibiting action of piperonyl cyclonene on the degradation of DDT. Winteringham, Loveday and Harrison (1951) obtained the same results with the bromine analog of DDT. If ability of resistant flies to survive is, in part, a function of detoxification of DDT, then inhibition of this process should show a quantitative relationship to mortality.

To obtain quantitative data on this subject, a series of tests was designed to determine: (1) the amounts of DMC in combination with a fixed dosage of DDT required to produce varying levels of mortality; (2) the effect of these amounts of DMC upon the degradation of DDT; and (3) the correlation, if any, between inhibition of detoxification and mortality. The results of three tests with groups of

100 flies per test shown in Table VI make such a correlation possible. Per cent inhibition was calculated as follows:

$$\frac{\text{Amount of DDE recovered from application of DDT and DMC}}{\text{Amount of DDE recovered from application of DDT alone}} \times 100 = X$$

$100 - X =$ per cent inhibition.

It may be noted that as the dosage of DMC was increased there was a corresponding increase in per cent inhibition of DDE formation and, consequently, an increase in mortality. It is also evident that complete inhibition was not essential for 100 per cent mortality of resistant flies.

TABLE VI

*The effect of DMC on inhibition of conversion of DDT to DDE and on mortality of adult female resistant house flies. Extractions made 24 hours after application. DMC separated chromatographically*

| Applied micrograms/fly | | Recovered* | | | | Per cent inhibition | Per cent mortality |
|---|---|---|---|---|---|---|---|
| | | DDT | | DDE** | | | |
| DDT | DMC | µg./fly | % of DDT applied | µg./fly | % of DDT applied | | |
| 0.65 | 0.0 | 0.0 | 0.0 | 0.51 | 78.4 | — | 0.0 |
| 0.65 | 0.06 | 0.06 | 9.4 | 0.41 | 63.0 | 19.6 | 2.0 |
| 0.65 | 0.13 | 0.11 | 16.9 | 0.38 | 58.4 | 25.5 | 14.0 |
| 0.65 | 0.32 | 0.18 | 27.7 | 0.32 | 49.2 | 37.2 | 25.0 |
| 0.65 | 0.65 | 0.29 | 44.6 | 0.26 | 40.0 | 49.0 | 50.0 |
| 0.65 | 1.30 | 0.35 | 53.8 | 0.18 | 27.7 | 64.7 | 72.0 |
| 0.65 | 3.25 | 0.45 | 69.2 | 0.12 | 18.4 | 76.5 | 92.0 |
| 0.65 | 6.50 | 0.50 | 76.9 | 0.08 | 12.3 | 84.3 | 100 |

* External rinses showed no DDT or DMC present.

** DDE figures are expressed as molecular equivalents of DDT.

## DISCUSSION

Synergism has been defined in many different ways; however, most definitions agree on one common point, *i.e.*, that the physiological effect of the substances in a mixture is significantly greater when used together than the summation of their individual effects. Bliss (1939) suggests that when the constituents of a mixture act independently, its toxicity does not depend upon the relative proportions of the components but only upon their inherent potencies. Synergistic action, in contrast, involves the ratio of one component to the other.

A conspicuous feature of Figure 1 is that as increasing amounts of DMC are added to DDT the probit-log dosage lines move toward smaller dosages of DDT and, in addition, become successively steeper. This process continues until a point is reached beyond which further addition of the synergist, within limits, causes either no increase or a slight decrease in activity. As seen from Figure 2, the addition of a small amount of DMC caused a significant drop in the amount of DDT required for a given mortality, whereas with large dosages of the adjuvant the effect was much less pronounced, as indicated by the hyperbolic nature of the curves. This reduction in DDT was roughly proportional to the mol fraction of DMC over a limited range of dosages, until the DMC was present in approximately equimolecular proportions with DDT. Figure 2 indicates that but little could be gained by further addition of DMC beyond this point; hence, by visual inspection, it may be reasonable to suppose that the 1 : 1 ratio is the optimum ratio of DDT : DMC.

It may also be noted (Fig. 1) from the data for the $LD_{50}$ and the $LD_{90}$ with DDT alone, and those for DDT plus the maximum amount of DMC, that the resistance of the Roberds strain may, under experimental laboratory conditions, be reduced to the extent of about 98 per cent (from 17.0 gamma to 0.40 gamma DDT per fly for the $LD_{50}$, and from 56.0 gamma to 0.58 gamma per fly for the $LD_{90}$). It should be borne in mind that these values are calculated in terms of amounts of DDT applied. Since the rate of absorption of DDT by house flies does not increase proportionally to increasing dosages by topical application (Fig. 3), the percentage reduction of resistance, if calculated on the basis of DDT absorbed, would be somewhat lower than 98 per cent. In no case, however, was it possible to reduce the resistance of the Roberds strain completely to the level of the susceptible strain ($LD_{50}$ approximately 0.30 microgram per fly).

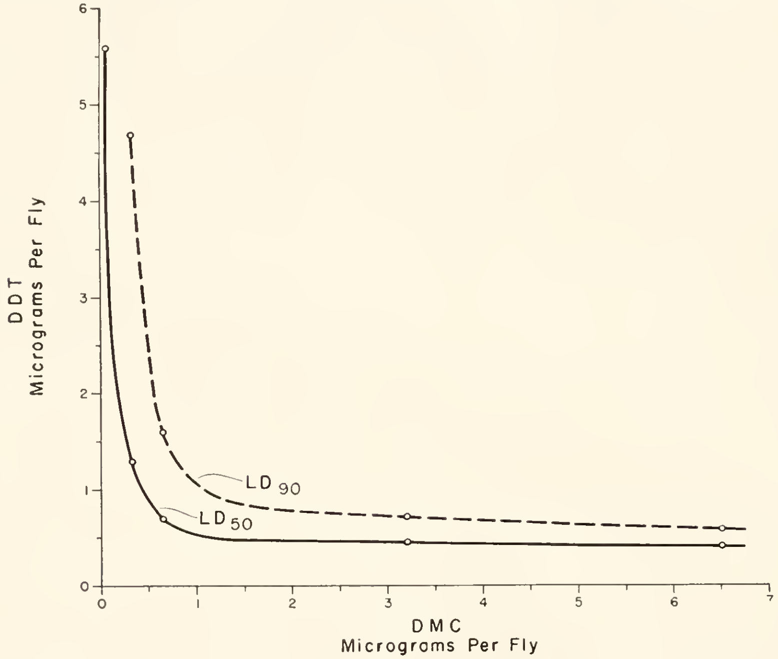


FIGURE 2. Curve relating dosage of DDT with dosage of DMC for 50 per cent and 90 per cent mortality of adult female house flies (Roberds strain).

There is reason to believe that synergistic compounds do not all act in the same manner, nor do they affect the same physiological system *in vivo*. It has been shown by various workers (Lindquist, Madden and Wilson, 1947; Wilson, 1949; Chamberlain, 1950; Perry and Hoskins, 1951a; and others) that pre-treating house flies with synergists followed by application of pyrethrins or DDT at different intervals did not materially affect the resultant mortalities as compared with simultaneous application of both compounds. In the present case, however, a marked reduction in activity resulted when applications of DMC and DDT were separated (Table III). This may indicate a different mechanism of synergistic action from those mentioned above.

The data of Tables IV and V show that DMC is rapidly metabolized by living flies. Thus, in 24 hours, from 50 to 70 per cent of the amount of DMC applied is excreted as DDA, and from 10 to 15 per cent is retained in the body either unchanged or in the form of DME. When sub-lethal amounts of DDT-DMC combinations are applied, both DDE and DDA are formed in substantial quantities. Lethal dosages of DDT-DMC, on the other hand, yield large amounts of DDT, small amounts of DDE and only traces of DDA. As a typical example, application of 0.3 gamma DDT and 0.3 gamma DMC per fly yielded in 24 hours 0.07 gamma DDT, 0.16 gamma DDE and 0.18 gamma DDA per fly, and the resulting mortality was 0–10 per cent. On the other hand, application of 0.6 gamma DDT plus 2.4 gamma DMC per fly yielded 0.36 gamma DDT, 0.13 gamma DDE and only traces of DDA, while the resulting mortality was 70–90 per cent.

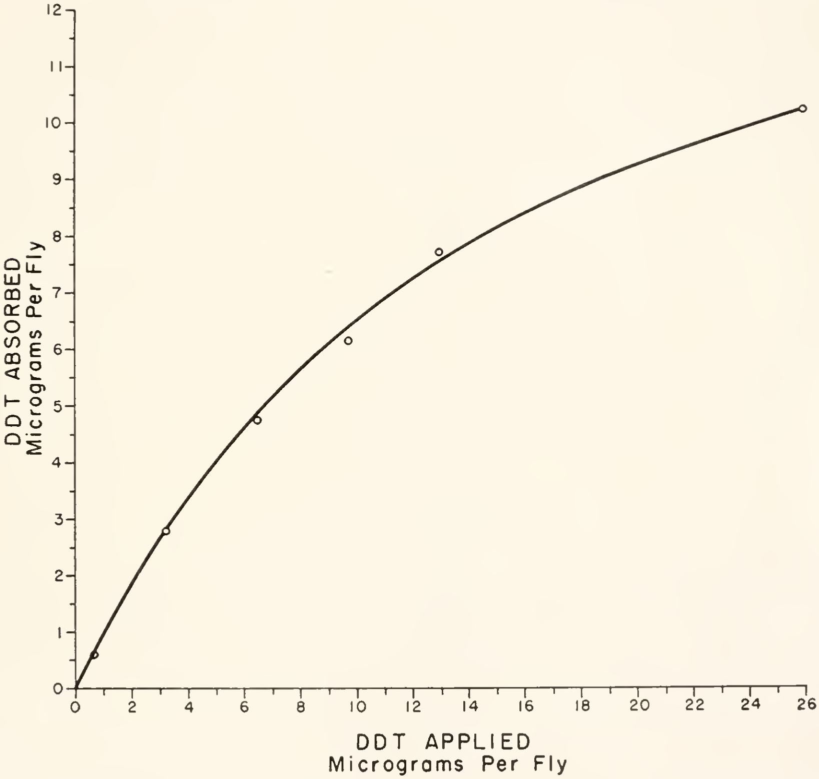


FIGURE 3. Curve relating amount of DDT absorbed in 24 hours with amount of DDT applied to adult female house flies (Roberds strain).

Since no satisfactory analytical methods for distinguishing between DMC and

DME are available, the role of DME in intermediary metabolism cannot be ascertained. However, on the basis of the following considerations, namely, (a) the relative ease of dehydration of DMC *in vitro*, (b) the conversion of DME to DDA *in vivo*, and (c) the fact that DME exhibits some synergistic activity with DDT, it is postulated that DME might be an intermediate product of DMC metabolism.

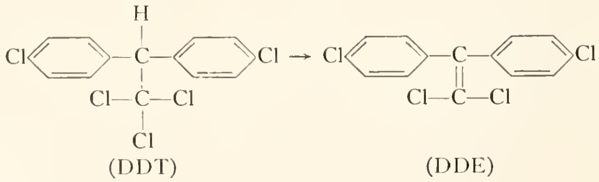
In attempting to elucidate the mechanism of synergistic action of DMC it is assumed that dehydrohalogenation of DDT, dehydration of DMC, as well as formation of DDA are enzymatic processes. As shown in Table VI a correlation exists between the extent of synergistic activity of DMC, as measured in terms of mortality, and the degree of inhibition of DDE formation.

Inhibitors of enzymatic reactions are usually of two types, competitive and non-competitive. In non-competitive inhibition, inactivation of the enzyme is independent of substrate concentration and depends only on concentration of inhibitor. In competitive inhibition, the inhibitor competes with the substrate for specific groups of the enzyme so that the apparent decrease in enzyme activity depends on the relative concentrations of both substrate and inhibitor (Lardy, 1949). In the present case it is apparent that inhibition is not of a non-competitive type, for the degree of activity is not independent of substrate (DDT) concentration (Fig. 1). Thus, for any constant dosage of inhibitor (DMC) the activity increased with increasing amounts of added substrate. Likewise, increased activity was manifested when increasing amounts of inhibitor were added to a constant substrate dosage.

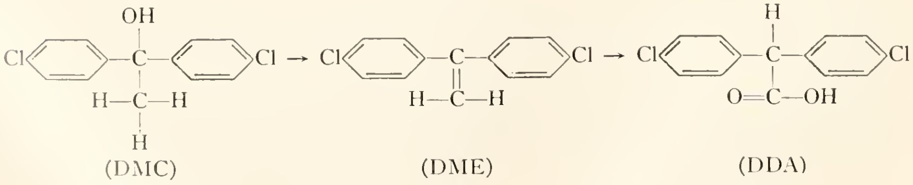
The great structural similarity between DMC and DDT and the knowledge that DMC is metabolized by living flies, plus the fact that greatest synergistic effect is manifested when DMC and DDT are applied simultaneously, suggest that inhibition is of a competitive type. This concept of "competition by displacement" is unlike the type of competitive inhibition by structurally related analogs of essential metabolites in which the added substrate may counteract the action of the inhibitor. In the present case the so-called metabolite or substrate is a poison which resistant flies are able to detoxify in varying quantities. Clearly, the addition of increasing amounts of this substrate cannot nullify the action of the inhibitor, but on the contrary, can only contribute to the injury already done.

The postulated reactions in this competitive system may be summarized as follows:

I



II



The quantitative relationship between inhibition of DDE formation and mortality of resistant house flies (Table VI) indicates that detoxification of DDT is a major factor in survival. If this were not the case, it would be difficult to explain why resistant flies should succumb to small quantities of DDT-DMC mixtures when they are able to tolerate large dosages of DDT.

DMC is non-toxic to house flies at fairly high dosages, and no observable symptoms are noted from application of DMC alone. Unless DMC causes the derangement of an unknown physiological process in the fly which might be associated with resistance, the above data point toward detoxification of DDT as a major factor in survival, and to inhibition of this process by DMC as a factor in mortality.

The nature and properties of the DDT-detoxifying mechanism have not yet been ascertained. However, the work of Sacktor (1951a, 1951b) on cytochrome oxidase in resistant and susceptible house flies suggests that this enzyme system might be associated with DDT-resistance. The relationship, if any, between cytochrome oxidase, DDT-detoxification and the inhibiting action of synergists has not been studied.

It is believed that the data presented in this report lend support to the hypothesis manifesting a specific competitive type of inhibition of DDT-detoxification as the mode of action of DMC as a DDT synergist.

The writers are indebted to Dr. R. W. Fay, Chief of the Insecticide Section, and to Dr. G. W. Pearce, Chief of the Chemistry Section, for many helpful suggestions and criticism throughout this study.

## SUMMARY

1. DMC has no insecticidal properties but markedly enhances the effectiveness of DDT against resistant house flies. The addition of a small amount of DMC causes a significant drop in the amount of DDT required for a given mortality.

2. Data interpolated from probit-log dosage lines for DDT alone and DDT plus DMC show that, under experimental laboratory conditions, the resistance of the Roberds strain can be markedly reduced.

3. Greatest synergistic effect is manifested when DMC and DDT are applied together, for separate application of the chemicals at 6-hour and 24-hour intervals shows a marked reduction in mortality.

4. DMC is rapidly metabolized by living flies and is excreted principally as a product tentatively identified as bis-(*p*-chlorophenyl) acetic acid (DDA). The compound 1,1-bis-(*p*-chlorophenyl) ethylene is suggested as being an intermediate product in DMC metabolism.

5. A correlation is shown between the extent of synergistic action and the degree of inhibition of DDT-detoxification in resistant flies.

6. Inhibition appears to be of a competitive type, the synergist competing with the insecticide for the mechanism of detoxification.

## LITERATURE CITED

Bliss, C. I., 1939. The toxicity of poisons applied jointly. *Ann. Appl. Biol.*, **26**: 585–615.

Chamberlain, R. W., 1950. An investigation on the action of piperonyl butoxide with pyrethrum. *Amer. J. Hygiene*, **52**: 153–183.

David, W. A. L., and P. Bracey, 1944. Activation of pyrethrins in fly sprays. *Nature,* **153**: 594–595.

Davidow, B., 1950. Isolation of DDT from fat. *J. Official Agr. Chemists,* **33**: 130.

Dove, W. E., 1947. Piperonyl butoxide, a new and safe insecticide for the household and field. *Amer. J. Trop. Med.,* **27**: 339–345.

Fullmer, O. H., and W. M. Hoskins, 1951. Effects of DDT upon the respiration of susceptible and resistant house flies. *J. Econ. Ent.,* **44**: 858–870.

Grummitt, O., A. C. Buck and E. I. Becker, 1945. 1,1-Di-(*p*-chlorophenyl)-ethane. *J. Amer. Chem. Soc.,* **67**: 2265.

Lardy, H. A., 1949. Respiratory enzymes. Burgess Publishing Co., Minneapolis, Minn.

Lindquist, A. W., A. H. Madden and C. H. Wilson, 1947. Pretreating house flies with synergists before applying pyrethrum sprays. *J. Econ. Ent.,* **40**: 426–427.

March, R. B., R. L. Metcalf and L. L. Lewallen, 1952. Studies on synergists for DDT against resistant house flies. *J. Econ. Ent.,* **45**: 851–860.

Munro, J. W., 1942. The entomology of commerce; insect pests and their control. *Analyst,* **67**: 155.

Page, A. B., and R. E. Blackith, 1949. Bioassay systems for pyrethrins, II. Mode of action of pyrethrum synergists. *Ann. Appl. Biol.,* **36**: 244–249.

Parkin, E. A., and A. A. Green, 1944. Activation of pyrethrins by sesame oil. *Nature,* **154**: 16–17.

Perry, A. S., and W. M. Hoskins, 1950. The detoxification of DDT by resistant house flies and inhibition of this process by piperonyl cyclonene. *Science,* **111**: 600–601.

Perry, A. S., and W. M. Hoskins, 1951a. Synergistic action with DDT toward resistant house flies. *J. Econ. Ent.,* **44**: 839–850.

Perry, A. S., and W. M. Hoskins, 1951b. Detoxification of DDT as a factor in the resistance of house flies. *J. Econ. Ent.,* **44**: 850–857.

Sacktor, B., 1951a. A comparison of the cytochrome oxidase activity of two strains of house flies. *J. Econ. Ent.,* **43**: 832–838.

Sacktor, B., 1951b. Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, *Musca domestica* L. *Biol. Bull.,* **100**: 229–243.

Schechter, M. S., S. B. Soloway, R. A. Hayes and H. L. Haller, 1945. Colorimetric determination of DDT. Color test for isolated compounds. *J. Ind. Eng. Chem., Anal. Ed.,* **17**: 704–709.

Sternburg, J., C. W. Kearns and W. N. Bruce, 1950. Absorption and metabolism of DDT by resistant and susceptible house flies. *J. Econ. Ent.,* **43**: 214–219.

Sumerford, W. T., Mary B. Goette, K. D. Quarterman and Sheely L. Schenck, 1951. The potentiation of DDT against resistant house flies by several structurally related compounds. *Science,* **114**: 6–7.

Sumerford, W. T., R. W. Fay, Mary B. Goette and A. Marian Allred, 1951. Promising DDT-synergist combinations for the control of resistant flies. *J. Nat. Malaria Soc.,* **10**: 345–349.

Wilson, C. S., 1949. Piperonyl butoxide, piperonyl cyclonene, and pyrethrum applied to selected parts of individual flies. *J. Econ. Ent.,* **42**: 423–428.

Winteringham, F. P. W., Patricia M. Loveday and A. Harrison, 1951. Resistance of house flies to DDT. *Nature,* **167**: 106.

# RECORDINGS OF HIGH WING-STROKE AND THORACIC VIBRATION FREQUENCY IN SOME MIDGES

OLAVI SOTAVALTA

*Department of Biology, Tufts College, Medford, Massachusetts* [1]

The highest known wing-stroke frequencies of insects have been recorded in male specimens of the dipterous families Chironomidae and Ceratopogonidae (Heleidae), where values of 1000/sec. have been obtained (Sotavalta, 1947). These recordings were made by registering acoustically the pitch of the flight-tone emitted by these insects during free flight. Until now, very few recordings of high wing-stroke frequencies have been obtained by the aid of other methods. Boettiger and Furshpan (1952) have obtained a wing-stroke frequency of 500/sec. in a chironomid midge, using electrostatic methods combined with cathode-ray oscillograph.

That still higher values of wing-stroke frequency can be produced, at least under artificial conditions, appears from the experiments reported below. The frequency determinations were made by three methods. The pitch of the flight-tone in free flight was determined acoustically; the flight-tone in free flight was also recorded by means of a microphone and high fidelity tape recorder, and with an accurate frequency check of 1000/sec. from a beat-frequency oscillator, transposed to recording film by means of a double-beam cathode-ray oscillograph; the thoracic vibration during fixed flight was registered by means of a piezo-electrical crystal pick-up and similarly recorded on tape and film. Male specimens of a small green species of *Chironomus* (s.lat.) and of a tiny species of *Forcipomyia* (Ceratopogonidae) were used in the experiments.

## EXPERIMENTS ON CHIRONOMUS: FLIGHT-TONE AND THORACIC VIBRATION FREQUENCIES

In order to record the flight-tone in free flight, the specimen was allowed to fly unmounted in a small glass chamber attached to the front of the microphone. The insect was stimulated to flight by letting it respond phototactically to a bright lamp near the glass chamber, or by shaking the glass chamber. The maximum duration of flight in the chamber obtained in this way was about 7–8 seconds. The flight-tone frequency recorded was about 600–650/sec. (Fig. 1), with occasional higher and lower values. Acoustical determinations were made before the experiment by allowing the insect to fly in a test-tube, the mouth of which was pressed against the ear. The general range of the flight-tone was determined as $d^{2}\sharp$–$e^{2}$ (622–659/sec.), and thus thoroughly agreed with the oscillographic recordings.

---

[1] This work was done while the author held a U. S. Government grant under Finnish Educational Exchange Program. The author wishes to express his gratitude to Prof. K. D. Roeder for valuable suggestions and assistance, and to Dr. Willis W. Wirth for the identification of the genus of *Forcipomyia.*

The thoracic vibrations were recorded in other specimens by mounting the insect on a stylus inserted in a crystal pickup (Roeder, 1951). Releasing the flight reflex by removing a platform from under the tarsi induced fixed flights for as long as 15 seconds, which could be recorded.

The thoracic vibration frequency was found to be about 520–580/sec. (Fig. 2a). A loudspeaker was turned on during the experiments, and it emitted a loud tone which thus was produced not by the wings but by the *thorax*. The frequency of this tone was determined acoustically as 554–587/sec. At the same time also the flight-tone produced by the wings was checked acoustically, and it was found in this case to have a pitch of $c^2\sharp$–$d^2$ (554–587/sec.), thus identical with the other determinations above.

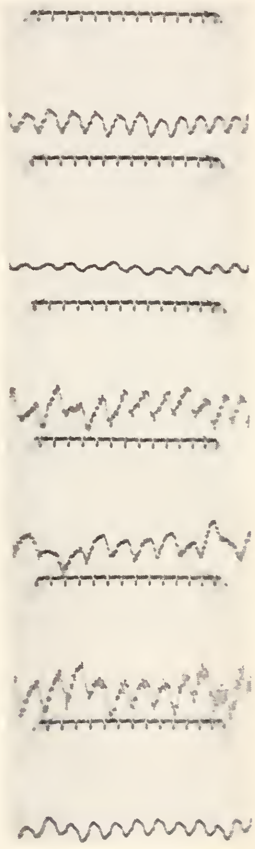


FIGURE 1. Flight-tone of *Chironomus* sp. and time-check 1000/sec. Frequency 600–680/sec.

The wings were then mutilated with transverse cuts, and a recording taken after each cut. First the left wing was cut to about half of its length. The thoracic vibration frequency (Fig. 2b), the frequency of the loudspeaker tone and of the flight-tone were again found to agree completely, being about 650–700/sec. ($e^2$–$f^2$).

The right wing was then cut to the equal half-length, and the frequency rose to about 830–880/sec. ($g^2\sharp$–$a^2$) (Fig. 2c). Both wings were then cut close to their bases, and the increased frequency was found to be about 1300–1400/sec. ($e^3$–$f^3$) (Fig. 2d).

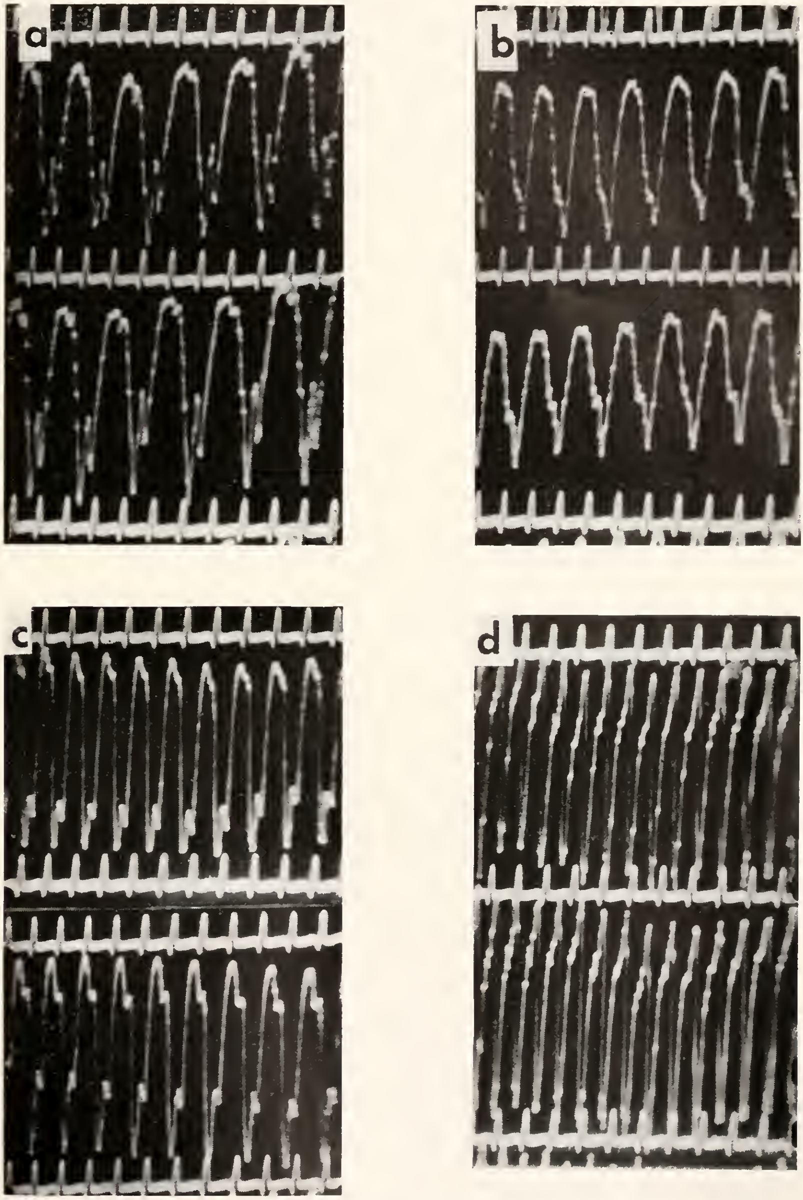


FIGURE 2. Thoracic vibrations of *Chironomus* sp. and time-check 1000/sec. (a) of an intact specimen, (b), (c) and (d) after three successive wing mutilations.

## EXPERIMENTS ON FORCIPOMYIA: FLIGHT-TONE FREQUENCIES

The specimen was allowed to fly unmounted in a glass chamber attached at the front of the microphone and flight was induced as in *Chironomus*. Also the flight-tone was checked acoustically before the experiment as above. The flight-tone frequency was found to be about 800–950/sec. ($g^2\sharp$–$b^2\flat$) (Fig. 3), and a complete agreement between the oscillographic and acoustic recordings was observed.

Unfortunately, it was not possible to mount a specimen of this species on the stylus, because even a slight heating of the mounting wax either killed the insect or, as it seemed, paralyzed the thoracic muscles. Any glue used for mounting did not adhere to the smooth and shiny surface of the thorax. Therefore no recording of the thoracic vibrations of these insects was possible.

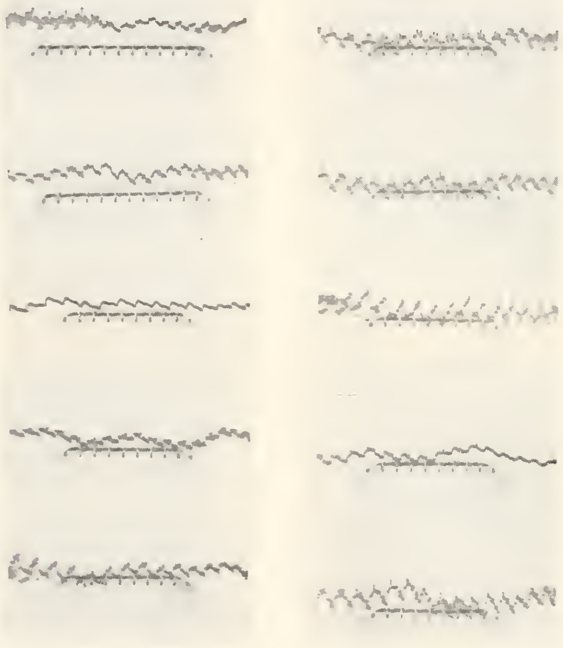


FIGURE 3. Flight-tone of *Forcipomyia* sp. and time-check 1000/sec. Frequency 800–950/sec.

Wing-mutilation experiments in this species were carried out with unmounted specimens. Since cutting the still more minute wings of such minute insects even with iridectomy scissors was extremely difficult without damaging the insect otherwise, only one experiment was successful. The wings were removed close to the base and the tone emitted determined acoustically by allowing the insect to "fly" in a small test-tube in front of an electric lamp. The flight-tone, which in the intact insect was $g^2\sharp$–$a^2$ (831–880/sec.), rose to $f^3$–$f^3\sharp$ (1397–1480/sec.). After this the test-tube with the midge was placed in an incubator at 37° C. for about 20–30 seconds. When it was taken out, the flight-tone was immediately determined. It proved to be $a^3$–$c^4\sharp$ (1760–2218/sec.). The midge, unfortunately, died so quickly after this experiment that no tape or film recording of these high frequencies was possible.

## Discussion

In order to produce a wing-stroke frequency of 1000/sec. the indirect flight muscles have to perform their complete cycle of contraction and relaxation in one msec. If the frequency is 2200/sec., this time is 0.45 msec., and thus both sets of indirect flight muscles perform 2200 contractions and relaxations during one second. Though it certainly is very doubtful whether there exist in nature insects which in an intact state possess a wing-stroke frequency comparable to the latter value, the producing of it artifically shows that the flight muscles are *capable* of such extreme performances under appropriate conditions. This fact also provokes a question about the minimum limit of time interval after which a muscle in general is able to repeat its contraction. Since the contraction frequency must be correlated with the intrinsic speed of the muscle, *i.e.,* with the ratio of contraction speed at zero load to fiber length (Hill, 1949), it follows that this limit, in turn, is correlated with the minimum limit of size in animals having well developed muscular organization. The problem whether motor nerves can transmit separate impulses with such a frequency is excluded because the indirect flight muscles of Diptera and of certain other insect orders possess a unique ability to maintain a rhythm of contractions without a motor nerve impulse preceding each contraction, as shown by Pringle (1949) and Roeder (1951). No microscopical insects, in the proper sense of the word, are known; therefore it is evident that in approaching this size limit the limit of contraction frequency also is approached. Since the speed of contraction also depends on load, even the most minute known insects cannot have an extremely high frequency of wing-strokes unless also the *relative* size of their wings is very small.

Since the energy output of a large and of a small insect is proportional to the cube of their linear dimensions, while the energy requirement is proportional to the fifth power of their linear dimensions, this leaves a margin in small insects within which the wing-stroke frequency can be increased (Sotavalta, 1952). Therefore a minute size is a *conditio sine qua non* for an extremely high wing-stroke frequency. In spite of this, however, there most likely appear additional factors which have a relatively greater significance as energy consumers in small than in large insects. The effect of drag in propulsion (*cf.* Sotavalta, 1952) and the dissipation of heat from the body to the air are proportional to the surface and therefore may necessitate a higher energy consumption in a small insect than predicted by the above reasoning.

## Summary

1. Experiments on recording flight-tones and thoracic vibrations in intact and wing-mutilated midges (*Chironomus, Forcipomyia*), using double-beam cathode-ray oscillograph and film and acoustic method, are reported.

2. In *Forcipomyia,* a wing-stroke frequency of 2218/sec. was the maximum value recorded, produced in a specimen with wings cut and exposed to high temperature.

3. Some aspects of the occurrence of such high frequencies in insects, correlated to the muscle contraction frequency in general, and of the energy consumption in small insects are discussed.

## LITERATURE CITED

Boettiger, E. G., and E. Furshpan, 1952. The recording of flight movements in insects. *Science,* **116**: 60–61.

Hill, A. V., 1949. The dimensions of animals and their muscular dynamics. Roy. Inst. Gt. Brit., Weekly Evening Meeting. Nov. 4, 1949. 24 pp. (Reprint.)

Pringle, J. W. S., 1949. The excitation and contraction of the flight muscles of insects. *J. Physiol.,* **108**: 226–232.

Roeder, K. D., 1951. Movements of the thorax and potential changes in the thoracic muscles of insects during flight. *Biol. Bull.,* **100**: 95–106.

Sotavalta, O., 1947. The flight-tone (wing-stroke frequency) of insects. *Acta Ent. Fenn.,* **4**: 1–117.

Sotavalta, O., 1952. The essential factor regulating the wing-stroke frequency of insects in wing mutilation and loading experiments and in experiments at subatmospheric pressure. *Ann. Zool. Soc. Vanamo,* **15**, 2: 1–67.

# A COMPARATIVE STUDY OF THE RESPIRATORY METABOLISM OF EXCISED BRAIN TISSUE OF MARINE TELEOSTS[1]

F. JOHN VERNBERG AND I. E. GRAY

*Department of Zoology, Duke University, Durham, North Carolina, and Duke Marine Laboratory, Beaufort, North Carolina*

In recent years several investigators have studied the respiratory metabolism of fish brain tissue. Fuhrman *et al.* (1944) studied the metabolism of excised brain of the large-mouthed bass, *Huro salmoides*, at graded temperature levels. In 1950 the phenomena of brain tissue metabolic adaptation to temperature in the polar cod, *Boreogadus saida*, and the golden orfe, *Idus melanotus*, were investigated by Peiss and Field. Freeman (1950), working with the goldfish, *Carassius auratus*, noted their brain metabolism during temperature acclimatization.

Certain physiological factors have been correlated with activity in marine fishes. Dawson (1933) showed that the more active fishes had a greater number of circulating immature erythrocytes than did less active species. Hall (1929, 1930) found that sluggish species not only consumed less oxygen but were capable of removing oxygen to lower tensions than could more active fishes. Hall and Gray (1929) and Gray and Hall (1930) found that in general the more active surface-feeding fishes had higher hemoglobin concentration and higher blood sugar level than did the sluggish bottom dwellers. Gray (1946, 1947) has pointed out a direct correlation between activity and the area of gill surface in marine teleosts.

It has seemed worthwhile to make a comparative study of the respiratory rate of excised brain tissue of marine teleosts to see if there might not be a correlation between brain metabolism and activity.

## MATERIALS AND METHODS

Fishes were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, N. C., and were maintained in the laboratory in aerated tanks supplied with running sea water. The procedures apply to all 17 species of marine fishes used in this study. Following exposure by cutting through the roof of the skull, all brain tissue anterior to the vagal lobes was removed, blotted quickly on filter paper, and weighed. The brain tissue was then ground in a dry mortar and taken up in a phosphate buffer of pH 7.5 (glass electrode) prepared by mixing 0.16 $M$ $KH_2PO_4$ and 0.121 $M$ $NaPHO_4$. Sufficient buffer was added to bring the volume to 3.0 ml. and then the brei was transferred to a Warburg flask. The center wall of the respirometer flask contained both 0.2 ml. of 10% KOH and filter paper wicks. Time between the death of the animal and the beginning of the 10-minute period of thermal equilibration was kept constant at 10 minutes. Manometric measurements were made in a bath maintained at 30° C. Readings were taken at 10-minute intervals and were carried out for a minimum of 60 minutes. Results

---

[1] Aided by a grant from the Duke University Research Council.

are expressed in terms of wet weight $Q_{O_2}$. Thus $Q_{O_2}$ (wet weight) denotes microliters of oxygen consumed per gram of wet weight per minute. The water content of the brain tissue of some species was determined by drying to a constant weight at 105° C.

## Results

The rate of oxygen consumption of brain brei preparations of 17 species of marine teleost fishes determined at 30° C. is shown in Table I. The fishes are arranged in the table, not in the order of their activity, but according to their rate of brain tissue oxygen consumption. Although a sharp dividing line is not evi-

Table I

*Oxygen consumption of brain breis of some marine teleost fishes*

| Species | No. of determinations | Mean $Q_{O_2}$ wet weight | Standard deviation |
|---|---|---|---|
| Group I: | | | |
| Menhaden<br>*Brevoortia tyrannus* | 8 | 14.19 | 3.66 |
| Mullet<br>*Mugil cephalus* | 10 | 13.52 | 1.46 |
| Sea trout<br>*Cynoscion nebulosus* | 9 | 12.53 | 2.66 |
| Sheepshead<br>*Archosargus probatocephalus* | 7 | 11.67 | 1.80 |
| Cutlass<br>*Trichiurus lepturus* | 3 | 11.03 | 1.86 |
| Group II: | | | |
| Sea bass<br>*Centropristus striatus* | 5 | 9.72 | 2.78 |
| Pinfish<br>*Lagodon rhomboides* | 24 | 9.30 | 0.91 |
| Croaker<br>*Micropogon undulatus* | 8 | 9.04 | 4.15 |
| Pigfish<br>*Orthopristis chrysopterus* | 15 | 8.95 | 1.11 |
| Silver perch<br>*Bairdiella chrysura* | 5 | 8.51 | 1.27 |
| Spot<br>*Leiostomus xanthurus* | 13 | 7.78 | 1.41 |
| Group III: | | | |
| Flounder<br>*Paralichthys dentatus* | 3 | 6.96 | 0.74 |
| Spiny boxfish<br>*Chilomycterus schoepfii* | 1 | 6.69 | — |
| Tongue fish<br>*Symphurus plagiusa* | 1 | 6.40 | — |
| Lizard fish<br>*Synodus faetens* | 2 | 6.01 | 0.01 |
| Toadfish<br>*Opsanus tau* | 13 | 5.57 | 1.16 |
| Hogchocker<br>*Achirus fasciatus* | 1 | 5.14 | — |

dent, the fishes are arbitrarily divided into three groups for convenience of discussion. Group I consists of those species which are the most active and are characterized by constant swimming movements. In Group II are those which seem to exhibit less activity than Group I, but are distinctly more active than the bottom-dwelling species of Group III.

Caution should be exercised in viewing too strictly the exact relationship of a few species because of the small number of determinations, especially in the case of the spiny boxfish which is not a typical bottom-dwelling species. However, these clearly demonstrated the tendency of the more sluggish fishes to have a slower rate of oxygen consumption of brain breis and therefore should be included. That the variation of metabolic rate of brain tissue was greater for certain species than for others is evident from the standard deviation obtained. Data for the croaker had a large standard deviation, whereas the standard deviation was small in the case of the pinfish.

When the weight of the pinfish (range 14.5–50 grams) was charted against the rate of oxygen consumption of brain tissue, no correlation was apparent. Similarly, when the length of the toadfish (range 12–40 centimeters) was plotted against the rate of oxygen consumption, no positive correlation was noted. Therefore, it would seem that within the size range of the fish used in this study, the rate of oxygen consumption of brain brei was independent of the weight or the length of the individual, and that the brain tissue of the younger or smaller individual of a species generally consumed about the same amount of oxygen per unit weight as the older or larger specimens.

The results of the brain tissue water-content determinations of 7 species of marine fishes are shown in Table II. In general, the water content is similar in all of the species examined.

TABLE II

*Brain tissue water content of 7 species of marine fishes*

| Species | No. of determinations | Average | Range |
|---|---|---|---|
| Mullet | 3 | 79.02% | 77.05–80.90% |
| Pigfish | 3 | 77.62% | 76.33–79.12% |
| Pinfish | 8 | 79.81% | 76.92–82.18% |
| Sea bass | 2 | 77.08% | 76.74–77.42% |
| Sheepshead | 2 | 76.28% | 75.08–77.48% |
| Spot | 7 | 78.58% | 75.00–80.83% |
| Toadfish | 4 | 78.27% | 77.50–79.12% |

## DISCUSSION

From the results of this study, a positive correlation is evident between the activity of marine teleost fishes and the oxygen consumption of brain breis.

Due to geographical difference in the distribution and relative abundance of fishes during the summer months at Beaufort, N. C., where the present study was made, and Woods Hole, Mass., where most of the early work reported in the literature was done, it is not possible to compare every species, but some species are common to both locations.

Hall and Gray (1929) and Gray and Hall (1930) found that those fishes which had the highest hemoglobin concentration and highest blood sugar level were considered to be the most active. Only two species of fishes investigated by them were used in the present investigation, the menhaden and the toadfish. The menhaden had a higher hemoglobin concentration and blood sugar level than the toadfish. In the present study, the rate of oxygen consumption of excised menhaden brain was distinctively higher than that of the toadfish. Dawson (1933) found the menhaden, as well as other active fishes, to have a greater number of immature circulating erythrocytes than the more sluggish forms, including the toadfish.

In respect to respiratory studies involving marine fishes, Hall and Gray (1929) point out that the menhaden has a higher rate of oxygen consumption than the more sluggish toadfish. Gray (1947) found that the menhaden has about 10 times more gill area than the toadfish per gram of body weight and 15 times more gill area per square centimeter of body surface. A comparison of other species shows that in general a rating of activity based on gill area per gram of weight or per square centimeter of body surface (Gray, unpublished) would agree with an activity rating based on oxygen consumption of brain tissue. A notable exception is the spiny boxfish but as stated previously only one determination was made on this species and more tests may alter the relative rating of this form.

Thus, when comparing the results of these various physiological indices of activity, as reported by other workers, with the results of the present study, it may be noted that there is a positive relationship between the various indices of activity and the data of this paper.

In general the results of the rate of brain tissue oxygen consumption in the present paper are similar to those values obtained by other workers (Fuhrman *et al.*, 1944; Peiss and Field, 1950; and Freeman, 1950). The influence of thermal acclimatization on the rate of oxygen consumption of brain tissue of fish was studied by Peiss and Field (1950) and Freeman (1950). They reported that at a common temperature the brain metabolism was highest for fish acclimatized at the lowest temperatures. No marked variation in respiration data was observed when comparing results of tests conducted in June with later determinations, as is evident in the case of the pinfish. The 24 determinations on this species were made at scattered intervals in June, July and August. The maximum-minimum water temperature taken in the Beaufort Channel from June 17 until August 20, 1952 showed an increase from readings of 80°–79° F. on June 17 to a high of 90°–87° F. on June 28. This was followed by a period of temperature decline and on July 5 the respective readings were 82°–76° F. During the month of August the highest reading was 84° F. and the lowest was 80° F. Thus it would seem that within the narrow temperature range at Beaufort during the period of this investigation no acclimatization effect of brain tissue was observed.

Although some workers have noted a decrease of tissue respiration with increase of body weight (Kayser, Le Breton and Schaeffer, 1925, brains of rats, pigeons and fowls; Hawkins, 1928, rat liver slices; Field *et al.*, 1937, rabbit lens; and Weymouth *et al.*, 1944, mid-gut gland of kelp crab), a similar relationship was not noted in the case of the pinfish and toadfish where the $Q_{O_2}$ appeared to be independent of body weight and body length. However, Grafe (1925) and Terroine and Roche (1925) found that any differences in tissue metabolism of large and small homoeothermic animals disappear when the tissues are removed from the body of the animal. Recently, Krebs (1950) reported that there is not a simple correlation between body size and $Q_{O_2}$ within the same species, and, in general, tissues of larger species have lower $Q_{O_2}$ values than homologous values of smaller species.

## Summary

1. The oxygen consumption of brain tissue of 17 species of marine teleost fishes was determined at 30° C.

2. A positive correlation exists between rate of oxygen consumption and activity of fishes. The more active fishes, like the menhaden, had a higher $Q_{O_2}$ than the sluggish fishes, such as the toadfish.

3. In general, when comparing results of this paper with various physiological indices of activity, a positive relationship was noted.

4. $Q_{O_2}$ of brain tissue of the pinfish and the toadfish were independent of body weight and body length.

## LITERATURE CITED

Dawson, A. B., 1933. The relative numbers of immature erythrocytes in the circulating blood of several species of marine fishes. *Biol. Bull.*, **64**: 33–43.

Field, J., II, E. G. Tainter, A. W. Martin and H. S. Belding, 1937. Studies on the oxygen consumption of the rabbit lens and the effect of 2–4 dinitrophenol thereon. *Amer. J. Ophthal.*, **20**: 779–794.

Freeman, J. A., 1950. Oxygen consumption, brain metabolism and respiratory movements of goldfish during temperature acclimatization, with special reference to lowered temperatures. *Biol. Bull.*, **99**: 416–424.

Fuhrman, F. A., Nell Hollinger, J. M. Crismon, J. Field, II and F. W. Weymouth, 1944. The metabolism of the excised brain of the largemouthed bass (*Huro salmoides*) at graded temperature levels. *Physiol. Zool.*, **17**: 42–50.

Grafe, E., 1925. Probleme der Gewebsatmung. *Deutsche. Med. Wchnschr.*, **51**: 640–642.

Gray, I. E., 1946. The relation between gill surface and activity in marine fishes. *Anat. Record*, **96**: 518.

Gray, I. E., 1947. The relation between gill surface and activity in marine fishes. *J. Elisha Mitchell Sci. Soc.*, **63**: 106.

Gray, I. E., and F. G. Hall, 1930. Blood sugar and activity in fishes with notes on the action of insulin. *Biol. Bull.*, **58**: 217–223.

Hall, F. G., 1929. The influence of varying oxygen tensions upon the rate of oxygen consumption in marine fishes. *Amer. J. Physiol.*, **88**: 212–218.

Hall, F. G., 1930. The ability of the common mackerel and certain other marine fishes to remove dissolved oxygen from sea water. *Amer. J. Physiol.*, **93**: 417–421.

Hall, F. G., and I. E. Gray, 1929. The hemoglobin concentration of the blood of marine fishes. *J. Biol. Chem.*, **81**: 589–594.

Hawkins, J. A., 1928. The metabolism of tissue from rats of different ages. *J. Gen. Physiol.*, **11**: 645–647.

Kayser, Charles, Elaine Le Breton and G. Schaeffer, 1925. Grandeur de la respiration des tissues et masse active au cours du développement des organismes. *C. R. Acad. Sci.* (Paris), **181**: 255–257.

Krebs, H. A., 1950. Body size and tissue respiration. *Biochim. et Biophys. Acta*, **4**: 249–269.

Peiss, C. N., and J. Field, 1950. The respiratory metabolism of excised tissues of warm- and cold-adapted fishes. *Biol. Bull.*, **99**: 213–224.

Terroine, E., and J. Roche, 1925. Production calorique et respiration des tissues *in vitro* chez les homeothermes. *C. R. Acad. Sci.* (Paris), **180**: 225–227.

Weymouth, F. W., J. M. Crismon, V. E. Hall, H. S. Belding and J. Field, II, 1944. Total and tissue respiration in relation to body weight: A comparison of the kelp crab with other crustaceans and with mammals. *Physiol. Zool.*, **17**: 50–71.

# INDEX

ACIDS, organic, cortical response of sea urchin egg to, 216.
Action of plant juices in chemotropism of Rhizopus, 100.
Activity in a crustacean ganglion. I. Cardio-inhibition and acceleration in Panulirus argus, 156.
Activity of cholinesetrase, effect of various suspension media on activity of, 323.
ALFERT, MAX. *See* DANIEL MAZIA, 57.
Algae as food for oyster larvae, 334.
Algae, respiration and iodine uptake in, 138.
Allylthiourea, effect of on histology of salamander thyroid and pituitary, 250.
Amino acids, treatment of sea urchin gametes with, 224.
Anaerobiosis in snails, 301.
Analysis of cortical response of sea urchin egg to reagents, 210, 216.
Analysis of molting process in the fiddler crab, Uca pugilator, 115.
Anatomy of Phoronopsis, 182.
Androgenetic hybrids, frog, competence of gastrula ectoderm in, 68.
Annual cycle in reproduction of Phoronopsis, 182.
Anticoagulants of vertebrate blood, reactions of insect hemolymph to, 372.
Aplexa nitens, oxygen consumption and oxygen tension in, 301.
Arbacia eggs, inhibition of cell division in, 263.
Arbacia punctulata, treatment of gametes of with metal-chelating agents, 224.
Arthropods, blood coagulation in, 372.
Ascophyllum, respiration and iodine uptake in, 138.
ATP and reversal of cell division inhibition in eggs, 263.
Australorbis glabratus, oxygen consumption and oxygen tension in, 301.
Azide and cell division inhibition in Arbacia eggs, 263.

B-MERCAPTOETHYLAMINE as protective agent against x-irradiation damage to Spisula gametes, 197.
BARNETT, ROBERT C. Cell division inhibition of Arbacia and Chaetopterus eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds, 263.
Behavior responses of white-footed mice in response to temperature changes, 87.
BLISS, DOROTHY E. Endocrine control of metabolism in the land crab, Gecarcinus lateralis (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs, 275.
Blood coagulation in arthropods. III. Reactions of insect hemolymph to coagulation inhibitors of vertebrate blood, 372.
BODINE, J. H., AND W. L. WEST. Carbohydrate metabolism of the developing egg and embryo, 1.
Body temperatures of white-footed mice in relation to environmental temperature and heat and cold stress, 87.
BONNER, JOHN TYLER, AND EVELYN B. FRASCELLA. Variations in cell size during the development of the slime mold Dictyostelium discoideum, 297.
Brain tissue of marine teleosts, respiratory metabolism of, 445.
VON BRAND, THEODOR, AND BENJAMIN MEHLMAN. Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some fresh water snails, 301.
BREWER, PHILIP A. *See* DANIEL MAZIA, 57.
Bromphenol blue, mercuric, staining and measurement of protein with, 57.
Brood production in Xiphophorus, 240.
BUCKNER, ANNETTE J. *See* ALBERT S. PERRY, 426.

CA-45, use of in measuring calcium uptake and turnover in oysters, 398.
Carbohydrate metabolism of the developing egg and embryo, 1.
Cardio-inhibition and acceleration in ganglion of Panulirus, 156.
CARLSON, J. GORDON, NYRA G. HARRINGTON AND MARY ESTHER GAULDEN. Mitotic effects of prolonged irradiation with low-intensity gamma rays on the Chortophaga neuroblast, 313.
Carnitine, studies on distribution of, 359.
Cell division inhibition of Arbacia and Chaetopterus eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds, 263.

Cell size, variations in during development of slime mold, 297.

Chadwick, L. E., J. B. Lovell and V. E. Egner. The effect of various suspension media on the activity of cholinesterase from flies, 323.

Chaetopterus eggs, inhibition of cell division in, 263.

Chaetopterus pergamentaceus, treatment of gametes of with metal-chelating agents, 224.

Chelating agents, metal, treatment of sea urchin gametes with, 224.

Chemicals, effects of on a schooling fish, 28.

Chemotropism in Rhizopus nigricans. II. The action of plant juices, 100.

Child, C. M. Indicator gradient patterns in oöcytes and early developmental stages of echinoderms: A reexamination, 12.

Chironomus, wing-stroke and thoracic vibration frequency in, 439.

Choleinate, sodium, cortical response of sea urchin egg to, 210.

Cholinesterase activity, effect of various suspension media on, 323.

Chortophaga, effect of low-intensity gamma radiation on mitosis in neuroblasts of, 313.

Ciliates, fibrillar system of, 408.

Clam, effects of x-irradiation on germ cells of, 197.

Clam, reproductive cycle in, 146.

Cleavage of x-irradiated Spisula eggs, 197.

Coagulation, blood, in arthropods, III., 372.

Cold stress in relation to body temperatures of white-footed mice, 87.

Color changes in Uca pugilator, 115.

Comparative study of the respiratory metabolism of excised brain tissue of marine teleosts, 445.

Competence of gastrula ectoderm in androgenetic frog hybrids, 68.

Composition of the swimbladder gas in deep sea fishes, 75.

Control, endocrine, of metabolism in land crab, 275.

Cortical response of sea urchin egg, analysis of, 210, 216.

Crassostrea virginica, food and feeding of larvae of, 334.

Crassostrea virginica, studies on shell formation in, 394, 398.

Crustacean ganglion, activity in, 156.

Cupron, treatment of sea urchin gametes with, 224.

Cyanide and inhibition of cell division in Arbacia eggs, 263.

Cyprina islandica, reproductive cycle in, 146.

Cysteine as protective agent against x-irradiation damage to Spisula gametes, 197.

Cysteine protection against x-irradiation damage of Tetrahymena, 351.

Cytochemical staining and measurement of proteins with mercuric bromphenol blue, 57.

van Dam, L. *See* P. F. Scholander, 75.

Davis, Harry C. On food and feeding of larvae of the American oyster, C. virginica, 334.

DDT, mechanism of synergistic action of DMC with against resistant house flies, 426.

DEDTC, treatment of sea urchin gametes with, 224.

Deep sea fishes, composition of swimbladder gas in, 75.

Dendraster excentricus, gradient patterns in oöcytes and embryos of, 12.

Deposition of calcium in oyster shells, 398.

Dermestes vulpinus, carnitine content of, 359.

Desmognathus, histology of thyroid and pituitary of, after treatment with thyroid inhibitors, 250.

Detergents, cortical response of sea urchin eggs to, 216.

Developing egg and embryo, carbohydrate metabolism in, 1.

Development of frog hybrids, studies in, IV., 68.

Development of slime molds, variations in cell size during, 297.

Developmental stages of echinoderms, gradient patterns in, 12.

Dictyostelium discoideum, variations in cell size during development of, 297.

"Dilution effect" in sea urchin sperm, 224.

Dinitrophenol and inhibition of cell division in Arbacia eggs, 263.

Dispersal of schooling fish, effects of chemicals on, 28.

Distribution of vitamin $B_T$, studies on, 359.

Diurnal rhythms in fiddler crab, 115.

DMC, mechanism of synergistic action of with DDT against resistant house flies, 426.

DNA activity in x-irradiated Tetrahymena, 351.

Dogfish, reabsorption of urea by kidney of, 45.

Ecdysis in fiddler crab, analysis of, 115.

Echinarachnius parma, treatment of gametes of with metal-chelating agents, 224.

Echinoderm oöcytes and embryos, gradient patterns in, 12.

Ectoderm, gastrula, competence of in androgenetic frog hybrids, 68.
Effects of chemicals on a schooling fish, Kuhlia sandvicensis, 28.
Effect of various suspension media on the activity of cholinesterase from flies, 323.
Effects of x-irradiation on nuclease activity and respiration of Tetrahymena, 351.
Effects of x-irradiation on Spisula gametes, 197.
Effects on mitosis in grasshopper neuroblast of prolonged low-intensity gamma rays, 313.
Egg, sea urchin, cortical response of to stimulating reagents, 210, 216.
Eggs, Arbacia and Chaetopterus, inhibition of cell division in, 263.
Eggs of sea urchin, treatment of with metal-chelating agents, 224.
Egner, V. E. *See* L. E. Chadwick, 323.
Eichel, Herbert J., and Jay S. Roth. The effect of x-irradiation on nuclease activity and respiration of Tetrahymena geleii W., 351.
Elasmobranch kidney, studies on, 45.
Electron microscopy of ciliate fibrillar systems, 408.
Embryo, grasshopper, carbohydrate metabolism in, 1.
Embryology of Phoronopsis, 182.
Endocrine control of metabolism in the land crab, Gecarcinus lateralis (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs, 275.
Environmental temperature in relation to body temperature of white-footed mice, 87.
Enzymes in Tetrahymena, effects of x-irradiation on, 351.
Eyestalk extirpation, effect of on respiratory metabolism of land crab, 275.

FERTILIZATION membrane of sea urchin eggs, formation of in response to stimulating agents, 210.
Fertilization of Phoronopsis ovum, 182.
Fertilization of x-irradiated Spisula eggs, 197.
Fertilization-reaction, improvement of by treatment of sea urchin gametes with metal-chelating agents, 224.
Fibrillar systems of ciliates as revealed by electron microscope. I. Paramecium, 408.
Fiddler crab, analysis of molting process in, 115.
Filtration rate of smooth dogfish kidney, 45.
Fish, reproduction in, 240.
Fish, schooling, effects of chemicals on, 28.
Fishes, deep sea, composition of swimbladder gas in, 75.
Flies, effect of various suspension media on activity of cholinesterase from, 323.
Flies, house, mechanism of synergistic action of DMC with DDT against resistant, 426.
Food and feeding of larvae of American oyster, C. virginica, 334.
Forcipomyia, wing-stroke and thoracic vibration frequency in, 439.
Formation of shell, studies on, II., 394.
Formation of shell, studies on, III., 398.
Fraenkel, G. Studies on the distribution of vitamin $B_T$ (carnitine), 359.
Frascella, Evelyn B. *See* John Tyler Bonner, 297.
Fresh water snails, oxygen consumption and oxygen tension in, 301.
Frog hybrids, studies in development of, 68.

GAMETES of marine animals, x-irradiation of, 197.
Gametes of sea urchin, treatment of with metal-chelating agents, 224.
Gamma rays, low-intensity, effect of on mitosis in grasshopper neuroblast, 313.
Ganglion, crustacean, activity in, 156.
Gas, swimbladder, composition of in deep sea fishes, 75.
Gastrula ectoderm, competence of in frog androgenetic hybrids, 68.
Gaulden, Mary Esther. *See* J. G. Carlson, 313.
Gecarcinus lateralis, endocrine control of metabolism in, 275.
Germ cells of clam, effect of x-irradiation on, 197.
Glomerular filtration in smooth dogfish kidney, 45.
Glycerol, depressant action of on fly cholinesterase, 323.
Goitrogens, effects of on histology of salamander thyroid and pituitary, 250.
Gonads, annual cycle in, in Phoronopsis, 182.
Gradient patterns in echinoderm oöcytes and embryos, 12.
Gradients, respiratory, in Tubularia, 109.
Grasshopper embryo, carbohydrate metabolism in, 1.
Grasshopper neuroblast, effects of low-intensity gamma rays on mitosis in, 313.
Gray, I. E. *See* F. John Vernberg, 445.
Grégoire, Ch. Blood coagulation in arthropods. III. Reactions of insect hemolymph to coagulation inhibitors of vertebrate blood, 372.

GUYSELMAN, J. BRUCE. An analysis of the molting process in the fiddler crab, Uca pugilator, 115.

HARRINGTON, NYRA G. *See* J. G. CARLSON, 313.
Heat stress in relation to body temperatures of white-footed mice, 87.
Helisoma duryi, oxygen consumption and oxygen tension in, 301.
Hemicentrotus pulcherrimus, cortical response of eggs to stimulating reagents, 210, 216.
Hemolymph, insect, reactions of to coagulation inhibitors of vertebrate blood, 372.
HIATT, ROBERT W., JOHN J. NAUGHTON AND DONALD C. MATTHEWS. Effects of chemicals on a schooling fish, Kuhlia sandvicensis, 28.
HIRATA, ARTHUR. Studies on shell formation. II. A mantle-shell preparation for in vitro studies, 394.
Histology of thyroid and pituitary of salamanders treated with thyroid inhibitors, 250.
Hormones, crustacean, effect of sinus gland and eyestalk extirpation on, 275.
House flies, mechanism of synergistic action of DMC with DDT against, 426.
Hybridization of Xiphophorus, 240.
Hybrids, androgenetic frog, studies of competence of gastrula ectoderm in, 68.

IMPROVEMENT of fertilization-reaction in sea urchin eggs by treatment with metal-chelating agents, 224.
Indicator gradient patterns in oöcytes and early developmental stages of echinoderms: a reexamination, 12.
Indophenol reaction in echinoderm oöcytes and embryos, 12.
Inhibition of cell division in Arbacia and Chaetopterus eggs, 263.
Inhibition of nucleases in x-irradiated Tetrahymena, 351.
Inhibitors, coagulation, reactions of insect hemolymph to, 372.
Inhibitors, thyroid, effects of on histology of salamander thyroid and pituitary, 250.
Insect hemolymph, reactions of to coagulation inhibitors of vertebrate blood, 372.
Intracellular oxidation of indicators in echinoderm oöcytes and embryos, 12.
In vitro studies on shell formation, method for, 394.
Iodine uptake and respiration in Ascophyllum, 138.
Irradiation with low-intensity gamma rays, effects of on mitosis in grasshopper neuroblast, 313.

JODREY, LOUISE H. Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$, 398.

KELLY, SALLY. Respiration and iodine uptake in Ascophyllum, 138.
KEMPTON, RUDOLF T. Studies on the elasmobranch kidney. II. Reabsorption of urea by the smooth dogfish, Mustelus canis, 45.
Kidney, elasmobranch, studies on, 45.
Krebs cycle intermediates in reversal of cell division inhibition in Arbacia and Chaetopterus eggs, 263.
Kuhlia sandvicensis, effects of chemicals on schooling of, 28.

LAMP brush chromosomes, staining of with mercuric bromphenol blue, 57.
Land crab, endocrine control of metabolism in, 275.
Larvae, oyster, food and feeding of, 334.
Latitude, rate of water propulsion in Mytilus as a function of, 171.
Life-span of sea urchin sperm, prolongation of, 224.
Lobster, Bermuda spiny, activity in ganglion of, 156.
LOOSANOFF, VICTOR L. Reproductive cycle in Cyprina islandica, 146.
LOVELL, J. B. *See* L. E. CHADWICK, 323.
Lymnaea stagnalis, oxygen consumption and oxygen tension in, 301.
Lytechinus pictus, treatment of gametes of with metal-chelating agents, 224.

MACTRA (Spisula), effects of x-irradiation on gametes of, 197.
Magnesium, effect of on oxygen uptake of grasshopper embryo homogenates, 1.
Mammalian tissues, vitamin $B_T$ content of, 359.
Malonate and inhibition of division in Arbacia eggs, 263.
Mantle-shell preparation, for in vitro study of shell formation, 394, 398.
Mantle tissue of oyster, calcium turnover in, 398.
Marine gametes, x-irradiation of, 197.
Marine teleosts, comparative study of respiratory metabolism of excised brain tissue of, 445.

Matthews, Donald C. *See* Robert W. Hiatt, 28.

Mattson, Arnold M. *See* Albert S. Perry, 426.

Maturation of ovum in Phoronopsis, 182.

Maynard, Donald M., Jr. Activity in a crustacean ganglion. I. Cardio-inhibition and acceleration in Panulirus argus, 156.

Mazia, Daniel, Philip A. Brewer and Max Alfert. The cytochemical staining and measurement of protein with mercuric bromphenol blue, 57.

Measurement of calcium deposition in shell and calcium turnover in mantle tissue of oysters, 398.

Measurement of protein with mercuric bromphenol blue, 57.

Mechanism of synergistic action of DMC with DDT against resistant house flies, 426.

Mehlman, Benjamin. *See* Theodor von Brand, 301.

Melanoplus differentialis, carbohydrate metabolism in embryos of, 1.

Mercuric bromphenol blue, staining and measurement of protein with, 57.

Metabolism, carbohydrate, of developing egg and embryo, 1.

Metabolism of DMC by house flies, 426.

Metabolism, endocrine control of in land crab, 275.

Metabolism of excised teleost brain tissue, 445.

Metabolism of fresh water snails, 301.

Metal-chelating agents, treatment of sea urchin gametes with, 224.

Metz, Charles B., Dorothy R. Pitelka and Jane A. Westfall. The fibrillar systems of ciliates as revealed by the electron microscope. I. Paramecium, 408.

Mice, white-footed, body temperatures of, 87.

Microscopy of ciliate fibrillar systems, 408.

Midges, wing-stroke and thoracic vibration frequency in, 439.

Mitotic effects of prolonged irradiation with low-intensity gamma rays on the Chortophaga neuroblast, 313.

Mitotic inhibition in Arbacia and Chaetopterus eggs, 263.

Molds, chemotropism in, 100.

Molluscs, studies on shell formation in, 394, 398.

Molting process in fiddler crab, analysis of, 115.

Moore, John A., and Betty C. Moore. Studies in the development of frog hybrids. IV. Competence of gastrula ectoderm in androgenetic hybrids, 68.

Musca domestica, effect of various suspension media on activity of cholinesterase from, 323.

Mustelus canis, reabsorption of urea by kidney of, 45.

Mytilus californianus, rate of water propulsion in, as function of latitude, 171.

NAUGHTON, John J. *See* Robert W. Hiatt, 28.

Neuroblast, grasshopper, effect of low-intensity gamma rays on mitosis in, 313.

Neurospora, vitamin $B_T$ in, 359.

Nitrogen tension in swimbladder of deep sea fishes, 75.

Nuclease activity, effect of x-irradiation on in Tetrahymena, 351.

OOCYTES, gradient patterns in, 12.

Orthoptera, reactions of hemolymph of to vertebrate blood coagulants, 372.

Ova, effects of x-irradiation on, 197.

Ovum of Phoronopsis, maturation and fertilization of, 182.

Oyster, food and feeding of larvae of, 334.

Oxidation of indicator dyes in echinoderm öocytes and embryos, 12.

Oxine, treatment of sea urchin eggs with, 224.

Oxygen consumption and oxygen tension in snails, 301.

Oxygen consumption of excised brain tissue of marine teleosts, 445.

Oxygen tension and inhibition of cell division, 263.

PANULIRUS argus, cardio-inhibition and acceleration in ganglion of, 156.

Paramecium, electron microscopy of fibrillar system of, 408.

Patiria miniata, gradient patterns in öocytes and embryos of, 12.

Peromyscus, body temperatures of in relation to environmental temperatures and heat and cold stress, 87.

Perry, Albert S., Arnold M. Mattson and Annette J. Buckner. The mechanism of synergistic action of DMC with DDT against resistant house flies, 426.

Phase contrast studies of insect blood, 372.

Phenylthiourea, effects of on histology of salamander thyroid and pituitary, 250.

Phormia regina, carnitine content of, 359.

Phoronopsis viridis, reproduction in, 182.

Phosphate compounds and reversal of cell division inhibition in Arbacia and Chaetopterus eggs, 263.

Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. I. Response to sodium choleinate and wasp venom, 210.

Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. II. The propagating or non-propagating nature of the cortical changes induced by various reagents, 216.
Physiological aspects of reproduction in Xiphophorus, 240.
Pitelka, Dorothy R. *See* Charles B. Metz, 408.
Pituitary, histology of in salamanders treated with thyroid inhibitors, 250.
Plankton as food for oyster larvae, 334.
Plant juices, action of in chemotropism of Rhizopus, 100.
Pre- and post-anaerobic oxygen consumption in snails, 301.
Prolongation of life-span of sea urchin spermatozoa and improvement of the fertilization-reaction by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron), 224.
Propagating nature of cortical response of sea urchin egg, 216.
Propulsion in Mytilus as a function of latitude, 171.
Protandry in Phoronopsis, 182.
Protein, staining and measurement of with mercuric bromphenol blue, 57.
Protozoa, effect of x-irradiation on nuclease activity and respiration of, 351.
Pumping rates in Mytilus, 171.
Pyruvate protection against x-irradiation damage in Tetrahymena, 351.

RADIOACTIVE iodine, uptake of by algae, 138.
Radiocalcium, use of in measuring deposition and turnover of calcium in oyster, 398.
Radioresistance of Spisula gametes, 197.
Rana, studies in development of hybrids of, 68.
Rao, K. P. Rate of water propulsion in Mytilus californianus as a function of latitude, 171.
Rattenbury, Joan C. Reproduction in Phoronopsis viridis. The annual cycle in the gonads, maturation and fertilization of the ovum, 182.
Reabsorption of urea by smooth dogfish kidney, 45.
Reagents, cortical response of sea urchin egg to, 210, 216.
Recordings of high wing-stroke and thoracic vibration frequency in some midges, 439.
Redox dyes, intracellular oxidation of, in echinoderm oöcytes and embryos, 12.
Regeneration in Tubularia, 109.
Relations betwen pre- and post-anaerobic oxygen tension and oxygen consumption in some fresh water snails, 301.
Repellents, fish, 28.
Reproduction in Phoronopsis viridis. The annual cycle in the gonads, maturation and fertilization of the ovum, 182.
Reproduction in Xiphophorus maculatus, 240.
Reproductive cycle in Cyprina islandica, 146.
Resistant house flies, mechanism of synergistic action of DMC with DDT against, 426.
Respiration and iodine uptake in Ascophyllum, 138.
Respiration, effect of x-irradiation on, in Tetrahymena, 351.
Respiration of fresh water snails, 301.
Respiration studies on developing grasshopper embryos, 1.
Respiratory gradients in Tubularia, 109.
Respiratory metabolism of excised teleost brain tissue, 445.
Respiratory metabolism of sinusglandless and eyestalkless land crabs, 275.
Reversal of cell division inhibition in Arbacia and Chaetopterus eggs, 263.
Rhizopus nigricans, chemotropism in, 100.
RNA activity in x-irradiated Tetrahymena, 351.
Roth, Jay S. *See* Herbert J. Eichel, 351.
Rugh, Roberts. The x-irradiation of marine gametes. A study of the effects of x-irradiation at different levels on the germ cells of the clam, Spisula (formerly Mactra), 197.

SALAMANDERS, histology of pituitary and thyroid of, after treatment with thyroid inhibitors, 250.
Scholander, P. F., and L. van Dam. Composition of the swimbladder gas in deep sea fishes, 75.
Schooling fish, effects of chemicals on, 28.
Sea urchin egg, cortical response of, 210, 216.
Sea urchin eggs and sperm, treatment of with metal-chelating agents, 224.
Sealander, John A., Jr. Body temperatures of white-footed mice in relation to environmental temperature and heat and cold stress, 87.
Shell formation, studies on, II., 394.
Shell formation, studies on, III., 398.
Sinus gland extirpation, effect of on respiratory metabolism of land crab, 275.
Sinus gland-X organ complex, role of in molting process of fiddler crab, 115.
Size of cells, variations in during development of slime mold, 297.

Slime molds, variations in cell size during development of, 297.
Snails, oxygen consumption and oxygen tension in, 301.
Sodium choleinate, cortical response of sea urchin egg to, 210.
Sotavalta, Olavi. Recordings of high wing-stroke and thoracic vibration frequency in some midges, 439.
Spawning of Phoronopsis, 182.
Sperm, effects of x-irradiation on, 197.
Sperm of sea urchin, treatment of with metal-chelating agents, 224.
Spisula, effects of x-irradiation on gametes of, 197.
Staining of protein with mercuric bromphenol blue, 57.
Stadler, David R. Chemotropism in Rhizopus nigricans. II. The action of plant juices, 100.
Stimulating reagents, analysis of cortical response of sea urchin egg to, 210, 216.
Strongylocentrotus, cortical response of eggs of to stimulating reagents, 210, 216.
Strongylocentrotus, gradient patterns in oöcytes and embryos of, 12.
Strongylocentrotus, treatment of gametes of with metal-chelating agents, 224.
Studies on the development of frog hybrids. IV. Competence of gastrula ectoderm in androgenetic hybrids, 68.
Studies on the distribution of vitamin $B_T$ (carnitine), 359.
Studies on the elasmobranch kidney. II. Reabsorption of urea by the smooth dogfish, Mustelus canis, 45.
Studies on shell formation. II. A mantle-shell preparation for in vitro studies, 394.
Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$, 398.
Study of effects of x-irradiation on Spisula gametes, 197.
Sucrose, depressant action of on fly cholinesterase, 323.
Sugiyama, Masao. Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. I. Response to sodium choleinate and wasp-venom, 210.
Sugiyama, Masao. Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. II. The propagating or non-propagating nature of the cortical changes induced by various reagents, 216.
Suspension media, effect of on activity of cholinesterase from flies, 323.
Swimbladder gas, composition of in deep sea fishes, 75.
Synergistic action, mechanism of, of DMC with DDT against resistant house flies, 426.
Synthesis of carnitine in insects, 459.
Sze, L. C. Respiratory gradients in Tubularia, 109.

TELEOSTS, marine, comparative study of respiratory metabolism of excised brain tissue of, 445.
Temperature, body of white-footed mice, 87.
Temporal variations in histological appearance of thyroid and pituitary of salamanders treated with thyroid inhibitors, 250.
Tenebrio, use of in bio-assay of carnitine, 359.
Tetrahymena geleii, effect of x-irradiation on nuclease activity and respiration of, 351.
Thiourea, effects of on histology of salamander thyroid and pituitary, 250.
Thoracic vibration frequency in some midges, 439.
Thyroid, histology of in salamanders treated with thyroid inhibitors, 250.
Transplantation experiments in hybrid frog embryos, 68.
Treatment of sea urchin gametes with metal-chelating agents, 224.
Tubularia, respiratory gradients in, 109.
Tyler, Albert. Prolongation of life-span of sea urchin spermatozoa, and improvement of the fertilization-reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron), 224.

UCA pugilator, analysis of molting process in, 115.
Urea, reabsorption of by smooth dogfish kidney, 45.

VALLOWE, Henry H. Some physiological aspects of reproduction in Xiphophorus maculatus, 240.
Variations in cell size during development of slime mold, 297.
Venom, wasp, cortical response of sea urchin egg to, 210.
Vernberg, F. John, and I. E. Gray. A comparative study of the respiratory metabolism of excised brain tissue of marine teleosts, 445.
Versene, treatment of sea urchin eggs and sperm with, 224.

Vertebrate blood, reactions of insect hemolymph to coagulation inhibitors of, 372.
Vitamin $B_T$, studies on distribution of, 359.

WARBURG manometry of snails, 301.
Wasp-venom, cortical response of sea urchin egg to, 210.
Water propulsion, rate of as function of latitude in Mytilus californianus, 171.
West, W. L. *See* J. H. Bodine, 1.
Westfall, Jane A. *See* Charles B. Metz, 408.
Wheat, vitamin $B_T$ in, 359.
Wheeler, Ambrose J. Temporal variations in histological appearance of thyroid and pituitary of salamanders treated with thyroid inhibitors, 250.
Wing-stroke and thoracic vibration frequency in some midges, 439.

X-IRRADIATION, effect of on nuclease activity and respiration of Tetrahymena, 351.
X-irradiation of marine gametes. A study of the effects of x-irradiation at different levels on the germ cells of the clam, Spisula (formerly Mactra), 197.
Xiphophorus maculatus, reproduction in, 240.

YEAST, vitamin $B_T$ in, 359.
Yolk, carbohydrate content of in grasshopper egg and embryo, 1.

Volume 104 Number 1

104, 1953
acc. # 67880
31 aug 53

# THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Marine Biological Laboratory
LIBRARY
FEB 24 1953
WOODS HOLE, MASS.

**Editorial Board**

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

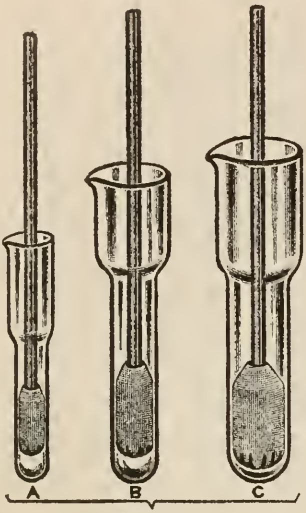
## FEBRUARY, 1953

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A. H. T. CO. SPECIFICATION

# TISSUE GRINDERS

**For the preparation of tissue homogenates for pathological and biochemical studies, tissue cultures, etc.**



4288-B.

***With Teflon pestle which minimizes powdered glass contamination of the homogenate and reduces heat formation.***

***Grinding vessel is of Pyrex brand glass precision bore tubing with 2 mm walls.***

***Grinding space is held to a close tolerance and remains practically constant in extended use.***

***Pestle is furnished with removable rod of either Stainless steel or aluminum.***

***All components can be autoclaved.***

**TISSUE GRINDERS, A.H.T. Co. Specification.** Consisting of piston-type Teflon pestle and replaceable grinding vessel of Pyrex brand glass. For the preparation of tissue homogenates for pathological and biochemical studies, tissue cultures, etc. Can be used for motor drive with rod inserted in the chuck of suitable slow speed stirrer.

Teflon has a smooth, waxy surface, is resilient, practically inert, and not wetted by water. Minimizes both heat generated in use and powdered glass in the homogenate, assuring maximal preservation of the accurate relation between pestle and grinding vessel over extended use, and providing reproducibility of cell lysis essential for quantitative comparison of homogenates from successive samples. See H. Brendler, *Science, July 20, 1951, p. 61.*

Grinding vessel is made of precision bore Pyrex brand glass tubing with walls 2 mm thick and with expanded upper portion for overflow. Clearance between chamber and pestle is held to approximately 0.13 mm. Pestle is furnished with removable rod of either Stainless steel or aluminum. Pestles and grinding vessels of the same size can be used interchangeably and all components can be autoclaved.

**4288-B. Tissue Grinders,** as above described, with Teflon pestle with removable rod of Stainless steel. Total capacity is approximately twice the capacity of the empty grinding chamber.

| Size | A | B | C |
|---|---|---|---|
| Capacity of grinding chamber without pestle, ml | 10 | 30 | 55 |
| Overall length of pestle, mm | 215 | 230 | 235 |
| **Each** | **7.65** | **12.65** | **17.75** |

10% discount in lots of 12, one size only.

NOTE—Can be furnished with Aluminum pestle rod at .22 less in price.

## ARTHUR H. THOMAS COMPANY

LABORATORY APPARATUS AND REAGENTS
WEST WASHINGTON SQUARE
PHILADELPHIA 5, PA.

Teletype Services: Western Union WUX and Bell System PH-72

# BIOLOGICAL ABSTRACTS

## Covers the World's Biological Literature

How do you keep abreast of the literature in your field? No individual possibly could accumulate and read all of the biological contributions in the original—yet some relatively obscure journal might publish a revealing paper on the very subject in which you are most interested.

*Biological Abstracts* now publishes concise, informative abridgments of all the significant contributions from more than 2,500 journals. As well as the complete edition, it also is published in nine low-priced sectional editions which are specially designed for individuals who are interested only in one or more closely related fields.

Production costs have increased to such an extent that the active support of all biologists is needed to maintain this important service. Write for full details and a sample copy of the sectional edition covering your field.

BIOLOGICAL ABSTRACTS
University of Pennsylvania
Philadelphia 4, Pa.

## MICROFILM SERVICE

◆

The Library of The Marine Biological Laboratory can supply microfilms of material from periodicals included in its list. Requests should include the title of the paper, the author, periodical, volume and date of publication.

◆

*Rates are as follows:* $1.00 for papers up to 50 pages, and $.10 for each additional 10 pages or fraction thereof.

## LANCASTER PRESS, Inc.

**LANCASTER, PA.**

☆

The experience we have gained from printing some sixty educational publications has fitted us to meet the standards of customers who demand the best.

We shall be happy to have workers at the MARINE BIOLOGICAL LABORATORY write for estimates on journals or monographs. Our prices are moderate.

# INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), short preliminary notes and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before they are refereed by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat; large illustrations may be rolled in a mailing tube. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

***Reprints.*** Authors will be furnished, free of charge, one hundred reprints without covers. Additional copies may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

---

## THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa.
under the Act of August 24, 1912.

# BIOLOGY MATERIALS

The Supply Department of the Marine Biological Laboratory has a complete stock of excellent plain preserved and injected materials, and would be pleased to quote prices on school needs.

* * *

## PRESERVED SPECIMENS

*for*

Zoology, Botany, Embryology, and Comparative Anatomy

## LIVING SPECIMENS

*for*

Zoology and Botany

including Protozoan and Drosophila Cultures, and Animals for Experimental and Laboratory Use.

## MICROSCOPE SLIDES

*for*

Zoology, Botany, Embryology, Histology, Bacteriology, and Parasitology.

*CATALOGUES SENT ON REQUEST*

* * *

Supply Department

**MARINE**
**BIOLOGICAL LABORATORY**
Woods Hole, Massachusetts

# CONTENTS

Page

BODINE, JOSEPH HALL, AND WILLIAM LIONEL WEST
Carbohydrate metabolism of the developing egg and embryo 1

CHILD, C. M.
Indicator gradient patterns in oöcytes and early developmental stages of echinoderms: A reexamination . . . . . . . . . . . 12

HIATT, ROBERT W., JOHN J. NAUGHTON AND DONALD C. MATTHEWS
Effects of chemicals on a schooling fish, Kuhlia sandvicensis 28

KEMPTON, RUDOLF T.
Studies on the elasmobranch kidney. II. Reabsorption of urea by the smooth dogfish, Mustelus canis . . . . . . . . . . . . . . . 45

MAZIA, DANIEL, PHILIP A. BREWER AND MAX ALFERT
The cytochemical staining and measurement of protein with mercuric bromphenol blue . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 57

MOORE, JOHN A., AND BETTY C. MOORE
Studies in the development of frog hybrids. IV. Competence of gastrula ectoderm in androgenetic hybrids . . . . . . . . . . . . . 68

SCHOLANDER, P. F., AND L. VAN DAM
Composition of the swimbladder gas in deep sea fishes . . . . . 75

SEALANDER, JOHN A., JR.
Temperatures of white-footed mice in relation to environmental temperature and heat and cold stress . . . . . . . . . . . . 87

STADLER, DAVID R.
Chemotropism in Rhizopus nigricans. II. The action of plant juices . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 100

SZE, L. C.
Respiratory gradients in Tubularia . . . . . . . . . . . . . . . . . . . . . . . . 109

Volume 104 Number 2

# THE
# BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

Marine Biological Laboratory
LIBRARY
APR 27 1953
WOODS HOLE, MASS.

APRIL, 1953

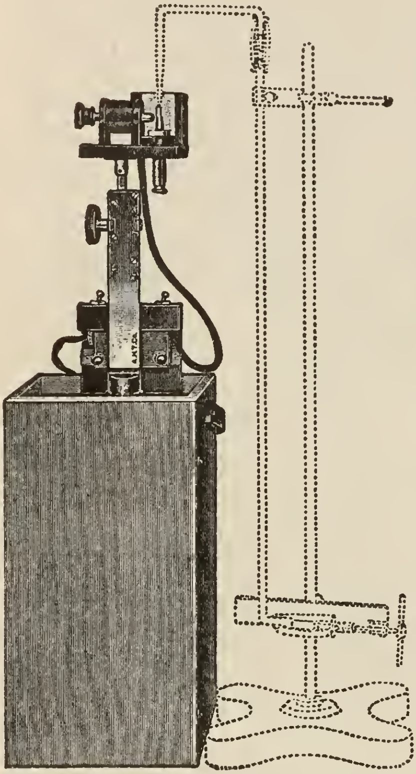
Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A.H.T. CO. SPECIFICATION

# MICRO TITRATION STAND

**For use in the Linderstrom-Lang-Holter technique for micro volumetric determinations**

---



**2463-F**

Showing Micro Titration Stand and Combination Storage Cabinet and Support in use with Linderstrom-Lang-Holter Burette.

**MICRO TITRATION STAND, A.H.T. Co. Specification,** with electromagnetic stirrer and illuminating unit, developed in cooperation with Dr. William L. Doyle. See David Glick, *Techniques of Histo- and Cytochemistry,* (New York, 1949), *pp 179–180.*

With self-starting, single-phase, synchronous motor; adjustable electromagnet enclosed in perforated metal cage, providing 180 magnetic impulses per minute, and a stirring bead, or "flea," which, when immersed in the reaction vessel and placed within the magnetic field, fluctuates between the bottom of the vessel and a point opposite the core of the electromagnet to produce efficient stirring of small liquid samples.

The illuminating unit diffuses the light and is provided with slots for filters 2 inches square. The Bakelite platform can be raised and lowered rapidly from heights between 11 and 17 inches by means of a rack and pinion operated by a handwheel; and contains an adjustable auxiliary stage, with 25 mm range, for raising and lowering the reaction vessel for precise control of stirring action. With switches for illuminating unit and electromagnet; and portable combination storage cabinet and support 16 inches high, for elevating the Stand when used with Linderstrom-Lang-Holter Burette.

**2463-F. Micro Titration Stand, A.H.T. Co. Specification,** as above described, complete with Storage Cabinet and Support, and with three reaction vessels with covers, one each single and double reaction vessel holders, 6 stirring beads, cord and plug, and detailed directions for use. For use on 115 volts, 60 cycles, a.c...... 179.64

**2463-G. Ditto,** but without reaction vessels, holders, stirring beads or storage cabinet. 152.35

***More detailed information regarding Burettes, Reaction Vessels, Volumetric Pipettes, and other accessories for use in the Linderstrom-Lang-Holter technique, sent upon request.***

---

## ARTHUR H. THOMAS COMPANY

### LABORATORY APPARATUS AND REAGENTS

WEST WASHINGTON SQUARE
PHILADELPHIA 5, PA.

**Teletype Services: Western Union WUX and Bell System PH-72**

# BIOLOGICAL ABSTRACTS

## COVERS THE WORLD'S BIOLOGICAL LITERATURE

How do you keep abreast of the literature in your field? No individual possibly could accumulate and read all of the biological contributions in the original—yet some relatively obscure journal might publish a revealing paper on the very subject in which you are most interested.

*Biological Abstracts* now publishes concise, informative abridgments of all the significant contributions from more than 2,500 journals. As well as the complete edition, it also is published in nine low-priced sectional editions which are specially designed for individuals who are interested only in one or more closely related fields.

Production costs have increased to such an extent that the active support of all biologists is needed to maintain this important service. Write for full details and a sample copy of the sectional edition covering your field.

BIOLOGICAL ABSTRACTS
UNIVERSITY OF PENNSYLVANIA
PHILADELPHIA 4, PA.

---

# MICROFILM SERVICE

◆

The Library of The Marine Biological Laboratory can supply microfilms of material from periodicals included in its list. Requests should include the title of the paper, the author, periodical, volume and date of publication.

◆

*Rates are as follows:* $1.00 for papers up to 50 pages, and $.10 for each additional 10 pages or fraction thereof.

---

# LANCASTER PRESS, Inc.

**LANCASTER, PA.**

*

THE EXPERIENCE we have gained from printing some sixty educational publications has fitted us to meet the standards of customers who demand the best.

We shall be happy to have workers at the MARINE BIOLOGICAL LABORATORY write for estimates on journals or monographs. Our prices are moderate.

# INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), short preliminary notes and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before they are refereed by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅝ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat; large illustrations may be rolled in a mailing tube. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

***Reprints.*** Authors will be furnished, free of charge, one hundred reprints without covers. Additional copies may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

## THE BIOLOGICAL BULLETIN

The Biological Bulletin is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa.
under the Act of August 24, 1912.

# *BIOLOGY MATERIALS*

**The Supply Department of the Marine Biological Laboratory has a complete stock of excellent plain preserved and injected materials, and would be pleased to quote prices on school needs.**

## PRESERVED SPECIMENS

*for*

**Zoology, Botany, Embryology, and Comparative Anatomy**

## LIVING SPECIMENS

*for*

**Zoology and Botany**

**including Protozoan and Drosophila Cultures, and Animals for Experimental and Laboratory Use.**

## MICROSCOPE SLIDES

*for*

**Zoology, Botany, Embryology, Histology, Bacteriology, and Parasitology.**

***CATALOGUES SENT ON REQUEST***

* * *

Supply Department

**MARINE**
**BIOLOGICAL LABORATORY**
Woods Hole, Massachusetts

# CONTENTS

Page

GUYSELMAN, J. BRUCE

An analysis of the molting process in the fiddler crab, Uca pugilator . . . . . 115

KELLY, SALLY

Respiration and iodine uptake in Ascophyllum . . . . . 138

LOOSANOFF, VICTOR L.

Reproductive cycle in Cyprina islandica . . . . . 146

MAYNARD, DONALD M., JR.

Activity in a crustacean ganglion. I. Cardio-inhibition and acceleration in Panulirus argus . . . . . 156

RAO, K. PAMPAPATHI

Rate of water propulsion in Mytilus californianus as a function of latitude . . . . . 171

RATTENBURY, JOAN C.

Reproduction in Phoronopsis viridis. The annual cycle in the gonads, maturation and fertilization of the ovum . . . . . 182

RUGH, ROBERTS

The x-irradiation of marine gametes. A study of the effects of x-irradiation at different levels on the germ cells of the clam, Spisula (formerly Mactra) . . . . . 197

SUGIYAMA, MASAO

Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. I. Response to sodium choleinate and wasp-venom . . . . . 210

SUGIYAMA, MASAO

Physiological analysis of the cortical response of the sea urchin egg to stimulating reagents. II. The propagating or non-propagating nature of the cortical changes induced by various reagents . . . . . 216

TYLER, ALBERT

Prolongation of life-span of sea urchin spermatozoa, and improvement of the fertilization-reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron) . . . . . 224

VALLOWE, HENRY H.

Some physiological aspects of reproduction in Xiphophorus maculatus . . . . . 240

WHEELER, AMBROSE J.

Temporal variations in histological appearance of thyroid and pituitary of salamanders treated with thyroid inhibitors . . . . . 250

Volume 104 Number 3

# THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

**Editorial Board**

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

JUNE, 1953

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A.H.T. Co PHILA U.S.A.
MICRO COVER GLASSES

Showing ½ oz. round wooden boxes and carton containing twelve ½ oz. boxes (6 ounces)

- **Cut from *machine drawn* flat strips of precision controlled thickness**

- ***NOW OFFERED AT REDUCED PRICES BECAUSE OF LOWER PRODUCTION COSTS***

## NON-CORROSIVE, RED LABEL
# MICRO COVER GLASSES

Cut, selected and packed in Philadelphia from the same excellent Chance micro sheet sold by us with great satisfaction continuously since 1902, improved from time to time, but now *mechanically drawn in strips*, which method of production offers the advantages of flatness and precision controlled thickness as compared with former method of hand blowing. Offered from stock in a wide variety of thicknesses, sizes and shapes—see pp. 887 and 888 of our catalogue. Outstanding characteristics are:

- ***High resistance to attack by moisture***
- ***Freedom from brittleness***
- ***Flatness***
- ***Precision controlled uniformity of thickness***

| | | Per ounce | Per oz. in 6 oz. *carton | Per oz. in 144 oz. assortments |
|---|---|---|---|---|
| 7020. | SQUARES, No. 1, 18 mm, 22 mm and 25 mm | 1.96 | 1.76 | 1.47 |
| | Ditto, No. 2 | 1.64 | 1.48 | 1.23 |
| 7022. | CIRCLES, No. 1, 18 mm, 22 mm and 25 mm | 3.12 | 2.81 | 2.34 |
| | Ditto, No. 2 | 2.68 | 2.41 | 2.01 |
| 7024. | RECTANGLES, Small, No. 1, 22 × 30 mm up to and including 22 × 50 mm | 1.96 | 1.76 | 1.47 |
| | Ditto, No. 2 | 1.64 | 1.48 | 1.23 |
| 7024. | RECTANGLES, Large, No. 1, 24 × 50 mm and 24 × 60 mm | 1.98 | 1.78 | 1.49 |
| | Ditto, No. 2 | 1.66 | 1.49 | 1.25 |
| 7024. | RECTANGLES, Large, No. 1, 35 × 50 mm up to and including 48 × 60 mm | 2.52 | 2.27 | 1.89 |
| | Ditto, No. 2 | 1.98 | 1.78 | 1.49 |

* One size, shape and thickness only.

# ARTHUR H. THOMAS COMPANY

LABORATORY APPARATUS AND REAGENTS
WEST WASHINGTON SQUARE PHILADELPHIA 5, PA.
Teletype Services: Western Union WUX and Bell System PH-72

# BIOLOGICAL ABSTRACTS

## Covers the World's Biological Literature

How do you keep abreast of the literature in your field? No individual possibly could accumulate and read all of the biological contributions in the original—yet some relatively obscure journal might publish a revealing paper on the very subject in which you are most interested.

*Biological Abstracts* now publishes concise, informative abridgments of all the significant contributions from more than 2,500 journals. As well as the complete edition, it also is published in nine low-priced sectional editions which are specially designed for individuals who are interested only in one or more closely related fields.

Production costs have increased to such an extent that the active support of all biologists is needed to maintain this important service. Write for full details and a sample copy of the sectional edition covering your field.

BIOLOGICAL ABSTRACTS
University of Pennsylvania
Philadelphia 4, Pa.

---

## MICROFILM SERVICE

◆

The Library of The Marine Biological Laboratory can supply microfilms of material from periodicals included in its list. Requests should include the title of the paper, the author, periodical, volume and date of publication.

◆

*Rates are as follows:* $1.00 for papers up to 50 pages, and $.10 for each additional 10 pages or fraction thereof.

---

## LANCASTER PRESS, Inc.

**LANCASTER, PA.**

☆

The experience we have gained from printing some sixty educational publications has fitted us to meet the standards of customers who demand the best.

We shall be happy to have workers at the marine biological laboratory write for estimates on journals or monographs. Our prices are moderate.

# INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), short preliminary notes and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before they are refereed by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat; large illustrations may be rolled in a mailing tube. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

***Reprints.*** Authors will be furnished, free of charge, one hundred reprints without covers. Additional copies may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

## THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa.
under the Act of August 24, 1912.

TOO TRANSPARENT **for ordinary microscopes**

BRILLIANT DETAIL **with AO**

# Phase Microscopes

Living organisms and tissues, pale or faded preparations, emulsions, plastics, and other materials are often too transparent to be seen by ordinary bright field microscopy.

Phase microscopy converts invisible differences in density in the specimen into images which are clearly seen and photographed.

AO pioneered today's methods of phase microscopy by developing numerous graduations and types of contrast for emphasizing different details and insuring that nothing is missed in examining transparent specimens.

Whether you prefer a standard contrast outfit which suffices for most applications, a small, special purpose outfit, or the most elaborate equipment — AO's long experience and complete selection of equipment are your best guarantee of satisfaction. For literature, write Dept. T–185.

American Optical
INSTRUMENT DIVISION
BUFFALO 15, NEW YORK

# CONTENTS

Page

BARNETT, ROBERT C.
Cell division inhibition of Arbacia and Chaetopterus eggs and its reversal by Krebs cycle intermediates and certain phosphate compounds 263

BLISS, DOROTHY E.
Endocrine control of metabolism in the land crab, Gecarcinus lateralis (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs ... 275

BONNER, JOHN TYLER, AND EVELYN BARBARA FRASCELLA
Variations in cell size during the development of the slime mold, Dictyostelium discoideum ... 297

VON BRAND, THEODOR, AND BENJAMIN MEHLMAN
Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some fresh water snails ... 301

CARLSON, J. GORDON, NYRA G. HARRINGTON AND MARY ESTHER GAULDEN
Mitotic effects of prolonged irradiation with low-intensity gamma rays on the Chortophaga neuroblast ... 313

CHADWICK, L. E., J. B. LOVELL AND V. E. EGNER
The effect of various suspension media on the activity of cholinesterase from flies ... 323

DAVIS, HARRY C.
On food and feeding of larvae of the American oyster, C. virginica ... 334

EICHEL, HERBERT J., AND JAY S. ROTH
The effect of x-irradiation on nuclease activity and respiration of Tetrahymena geleii W ... 351

FRAENKEL, G.
Studies on the distribution of vitamin $B_T$ (carnitine) ... 359

GRÉGOIRE, CH.
Blood coagulation in arthropods. III. Reactions of insect hemolymph to coagulation inhibitors of vertebrate blood ... 372

HIRATA, ARTHUR A.
Studies on shell formation. II. A mantle-shell preparation for in vitro studies ... 394

JODREY, LOUISE H.
Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and $Ca^{45}$ ... 398

METZ, CHARLES B., DOROTHY R. PITELKA AND JANE A. WESTFALL
The fibrillar systems of ciliates as revealed by the electron microscope. I. Paramecium ... 408

PERRY, ALBERT S., ARNOLD M. MATTSON AND ANNETTE J. BUCKNER
The mechanism of synergistic action of DMC with DDT against resistant house flies ... 426

SOTAVALTA, OLAVI
Recordings of high wing-stroke and thoracic vibration frequency in some midges ... 439

VERNBERG, F. JOHN, AND I. E. GRAY
A comparative study of the respiratory metabolism of excised brain tissue of marine teleosts ... 445

MBL/WHOI LIBRARY
WH 1AZ5 E